

Thesis for the Master's degree in chemistry

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***Screening and examination of
small molecules to find inhibitors
and synergists of the canonical
Wnt signaling pathway.***

60 study points

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Preface

This master thesis describes the result of my Master's degree in chemistry at the University of Oslo. The work has been carried out at Stefan Krauss' Cellular and Genetic Therapy Research Group (Institute of Microbiology, Rikshospitalet) between January 2008 and January 2009.

I started my Master's degree in January 2007 with the intention of carrying a thesis work on gene correction and Zinc Finger Nucleases, but changed the subject after one year since the main supervisor left the institute. When I started on the present thesis subject in January 2008, the aim was to find a small molecule that increased or decreased the binding between Wnt and Frizzled and to discuss the bindings in a chemical perspective. Unfortunately the paper from Stefan Krauss' Cellular and Genetic Therapy Research Group has not yet been published, and due to secrecy until publishing my master thesis could not reveal the structures.

I would like to thank Stefan Krauss for giving me the opportunity to work on this project. I would also specially want to thank my supervisor at the lab and my supervisor at the University of Oslo, Jo Waaler and Elsa Lundanes, for all their help, advise, encouragement and patience.

A warm thanks also to my coworkers and to family and friends. Especially my coworker and friend Eline Buchman for all her support and positive reinforcement, my sister Hege Janita Bråtesveen for making me soup for the long nights of writing, and for helping me with graphics. All my love to my boyfriend, Morten Skråmestø, for his proofreading, support and patience.

Oslo, April 11th 2010

Stine Maria Bråtesveen

Abbreviations and definitions

aa	amino acid
APC	adenomatous polyposis coli
ASCR	Academy of Sciences of the Czech Republic
ATCC	American Type Culture Collection
c/w	cells per well
CBP	cyclic AMP response element-binding protein
cDNA	complementary DNA
CK1	casein kinase 1
CK2	casein kinase 2
CRD	cysteine-rich domain
Dhh	Desert hedgehog
DlgA	Drosophila disc large tumor suppressor
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside triphosphates
DTT	Dithiothreitol
Dvl	Dishevelled
e.g.	<i>exempli gratia</i>
FBS	Foetal Bovine Serum
Frz	Frizzled
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3	glycogen synthase kinase 3
HDAC	Histone deacetylase
HEK	Human Embryonic Kidney
HSV-TK	herpes simplex virus thymidine kinase
Ihh	Indian hedgehog
IMG	Institute of Molecular Genetics
I κ Bs	inhibitors of κ B
LRP	low-density lipoprotein receptor-related proteins
MMTV	mouse mammary tumor virus
MQ	Milli-Q
mRNA	messenger RNA
NF- κ B	nuclear factor kappa B
NIH	The National Institutes of Health resource for stem cell research
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pen Strep, PS	penicillin streptomycin
PKA	protein kinase A
PKC	protein kinase C
pNF- κ B-luc	NF- κ B-luc plasmid
PP1	protein phosphatase 1
pRL-TK	<i>Renilla</i> plasmid
PSD95	Post synaptic density protein
pSTF	Super TOPFlash plasmid
Ptch	Patched
RNA	Ribonucleic Acid

RNases	ribonucleases
RT-PCR	reverse transcription-PCR
SAMP	Serine-Alanine-Methionine-Proline
SCF	Skp1-Cul1-F-box-protein
sFRP-1	secreted frizzled-related protein 1
Shh	Sonic hedgehog
Smo	Smoothened
STF	Super TOP-Flash
TCF/LEF	T-cell-specific transcription factor/lymphoid enhancer-binding factor 1
TGF- β	transforming growth factor beta
TNF- α	tumor necrosis factor α
TNKS	tankyrase
Trypsin-EDTA	trypsin-ethylenediaminetetraacetic acid
Wg	Wingless
wp	well plate
wt	wild type
zo-1	zonula occludens-1 protein
β -TrCP	β -transducin repeat-containing protein

Adherent cell lines:	Cells can be grown in suspension or adherent cultures. Adherent cells attach themselves to the surface inside the growth flask.
Cell signaling pathways:	Signaling pathways in between cells.
Conditioned medium:	Medium collected from a growing cell line which secret biologically active components, and these components will affect some cell functions.
Endogenous luciferase activity:	The luciferase activity inn the cell without activating the canonical Wnt signaling pathway with Wnt-3A conditioned medium.
Ligand:	Protein that binds to and activates a receptor (e.g. the ligand Wnt bind to the receptor Frz).
Plasmid:	A circular molecule of double stranded DNA that can be replicated inside the cell independent of the chromosomal DNA. A type of vector.
Primer:	A strand of nucleic acid (DNA or RNA) that is complementary to a given DNA sequence and serves as a starting point for DNA replication by the enzyme DNA polymerase.
Reporter gene:	Often simply called reporter. A transfected gene that produces a signal (e.g. fluorescence or bioluminescence) when expressed. The reporter gene is often attached to the regulatory sequence of a gene to quantify this expression.
Stem cell:	Unspecialized cells that are capable of self renewing through cell division, and they may differentiate into a wide range of specialized cells.
Synergist:	An agent (molecule) that increases the effect of another.
Transcription factor:	Protein that bind to regulatory units in the genome and control the expression of genes.
Transfection:	The process by which exogenous DNA or RNA is introduced into cells.
Vector:	A DNA molecule used to transfer exogenous DNA or RNA is introduced into cells.

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Abstract

From an *in silico* based screening of 10.000 compounds done by Andrew Voronkov prior to this master thesis, 256 small molecules have been examined to find compounds that acted as synergists or inhibitors by increasing or decreasing the binding between the ligand Wnt and the receptor Frizzled in the canonical Wnt signaling pathway. A few possible inhibitors and several possible activators were found and the structures of the latter were used for analogue development of 226 compounds which were also examined.

Compound C1321 was established as the most potent compound, with some analogues of compound C1321 showing similar or a somewhat less activating effect.

Compound C1321 was identified to be a synergist of the canonical Wnt signaling pathway with a relative activation up to 3500 % compared to controls and no activating effect without Wnt-3A present in the medium. This was established using the Luciferase Assay System with HEK293 STF cells and Wnt-3A conditioned medium or regular cell medium. The results was also confirmed by the Dual-Luciferase Reporter Assay System with transiently transfected HEK293 wt cells with STF and *Renilla* plasmids, and by Axin2 real-time RT-PCR on HEK293 wt cells. The specificity of compound C1321 for the canonical Wnt signaling pathway was explored by examination of the effect on the NF- κ B signaling pathway and the Sonic hedgehog signaling pathway. The Dual-Luciferase Reporter Assay System with HEK293 wt cells transiently transfected with *Renilla* and NF- κ B-luc plasmids and activated with TNF- α medium showed no activating effect on the NF- κ B signaling pathway. The Dual-Luciferase Reporter Assay System with Shh-Light2 cells and conditioned Shh-medium showed no activating effect on the Shh signaling pathway.

1 Introduction

1.1 *Stem cell research*

The history of stem cell research started already in the mid 1800s, with the discovery of cell propagation and differentiation, due to improved microscopes¹. The discovery of cells that generate different types of blood cells was done in the early 1900s², and the Russian histologist Alexander Maksimov came up with the term “stem cell” in 1908 at a congress of hematologic society in Berlin³. From the 1960s the stem cell research really took form, and the first bone marrow transplant on humans was performed in 1968⁴.

Stem cells are per definition unspecialized cells that are capable of self renewing through cell division, and they may differentiate into a wide range of specialized cells^{5,6}.

The potential in using stem cells for treatment and cure of many human diseases and disorders makes this a promising research field.

1.2 *Signaling pathways*

Inside unicellular organisms, metabolic pathways control the cells biological processes. Multicellular organisms are dependent on signaling pathways in between cells (cell signaling pathways) as well as signaling pathways inside the cells (metabolic pathways).

Some of the best known signaling pathways with the current overviews and pathway diagrams can be found on the internet pages of Cell Signaling Technology⁷. Four signaling pathways that are especially linked to development of the cell is the transforming growth factor beta (TGF- β) signaling pathway, the Notch signaling pathway, the Hedgehog signaling pathway, and the canonical Wnt signaling pathway.

1.3 The Wnt signaling pathway

Embryo development is a complex process that involves a variety of steps. Only a few different signal pathways are responsible for the multifaceted operation of embryo development, and the pleiotropic Wnt signaling pathway affects almost all steps; e.g. mesoderm induction^{8, 9}, neural induction^{10, 11}, and body axis formation^{12, 13}.

In mature organisms the Wnt signaling pathway is required for adult tissue maintenance^{14, 15, 16}. When the pathway is dysfunctional e.g. due to mutations, several human degenerative diseases and numerous cancers may occur. Alzheimer¹⁷, multiple sclerosis¹⁸, polycystic kidney disease¹⁹, type 2 diabetes²⁰, arthritis²¹, colon cancer¹⁶, lung cancer²² and breast cancer²³ are some examples.

The Wnt signaling pathway achieved its name from a gene in mouse (Int-1, discovered in 1982²⁴), and its orthologue in *Drosophila* (Wg, wingless, discovered in 1987)^{25, 26, 27}. Int-1 is a proto-oncogene that is activated by integration of mouse mammary tumor virus (MMTV) in mammary tumors²⁴.

Three different evolutionary conserved pathways that are attended with Wnt receptor activation exists, the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non canonical Wnt/Ca²⁺ pathway^{28, 29}. Of these the first to be explained was the canonical Wnt signaling pathway²⁹.

1.3.1 The canonical Wnt signaling pathway

One of the main players in the canonical Wnt signaling pathway is β -catenin, which regulates gene expression by direct interaction with transcription factors.

β -catenin, Figure 1, is a member of the armadillo family of proteins. The central area of β -catenin is a super helix of helices, containing 12 repeats of three helices each³⁰. These repeats, called “armadillo repeats”, are domains that are specialized for protein-protein binding, and β -catenin may therefore bind to a variety of complexes³⁰.

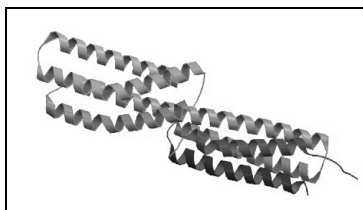


Figure 1 3D-structure of β -catenin³¹.

The canonical Wnt signaling pathway is well described. Figure 2 shows a simplified illustration of this pathway.

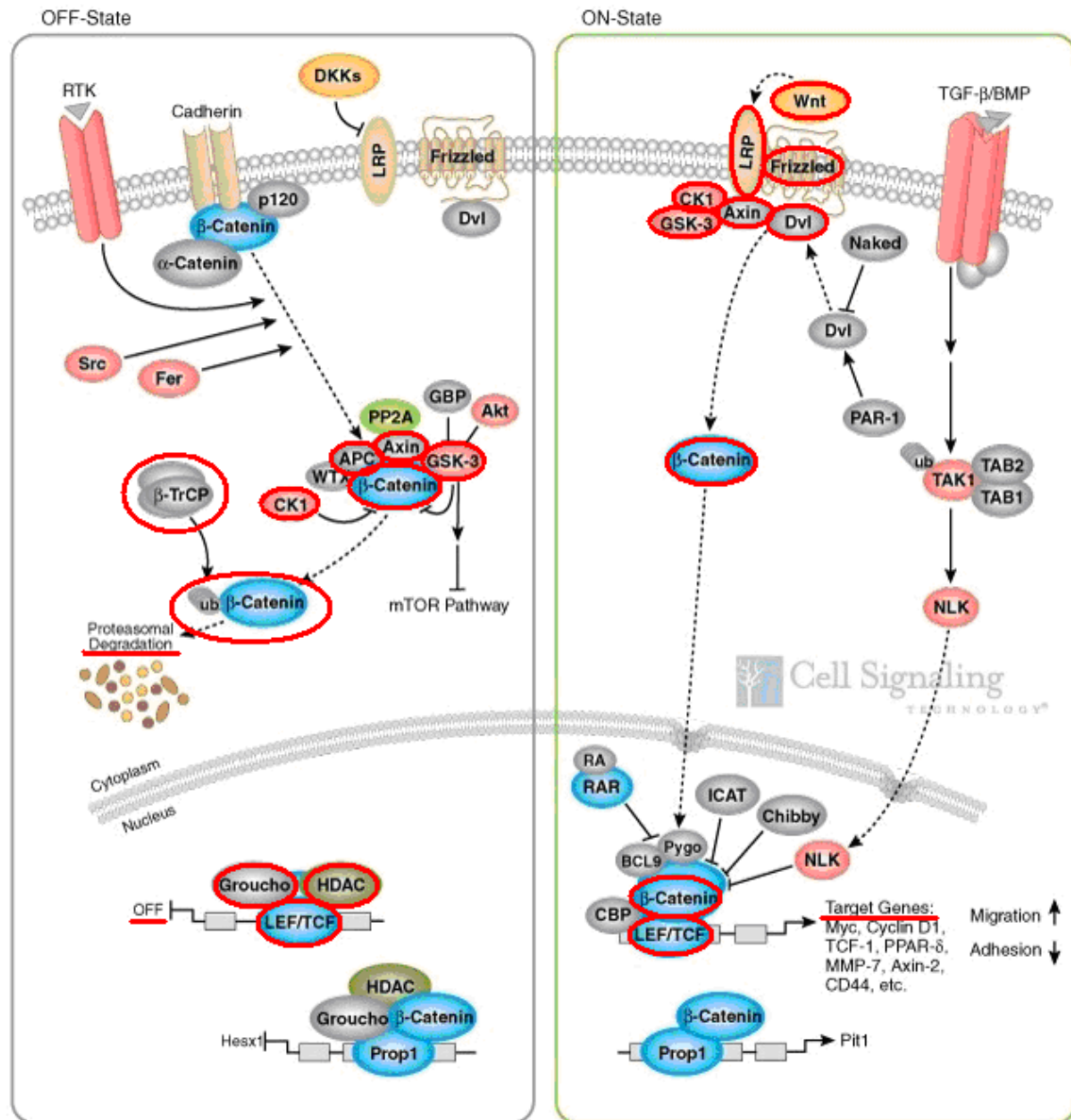


Figure 2 Simplified illustration of the canonical Wnt signaling pathway and some of the components³².

OFF-state: The destruction complex with the scaffolding proteins Axin and APC and the kinases GSK3 and CK1 phosphorylates β -catenin and targets it for β -TrCP, polyubiquitination and proteosomal degradation. The transcriptional repressors Groucho and HDAC bind to LEF/TCF and prevent transcription of target genes.

ON-state: The Wnt ligand binds to the receptor Frizzled and the co-receptor LRP. Dvl attach to the cell membrane and help aggregation and phosphorylation of LRP by CK1 and GSK-3. This phosphorylation of LRP will give Axin a higher affinity for LRP and the destruction complex will not be formed. β -catenin enters the nucleus and binds to LEF/TCF to activate transcription of target genes.

1.3.2 The canonical Wnt signaling pathway – OFF-State

In un-stimulated cells β -catenin will not enter the nucleus in significant amounts. This is mainly due to a destruction complex in the cytoplasm which phosphorylates β -catenin and targets it for ubiquitination and degradation³³.

The destruction complex is built up of a variety of different proteins. Some of the main components of this complex are the scaffolding proteins Axin and adenomatous polyposis coli (APC), and the serine/threonine kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) which phosphorylates β -catenin³³. These components of the destruction complex will be explained briefly later.

The phosphorylation of β -catenin targets it for a β -transducin repeats-containing protein (β -TrCP)^{34, 35}, which is a subunit of the Skp1-Cul1-F-box-protein (SCF) E3 ubiquitin ligase³⁶. This E3 ubiquitin ligase will contribute to a polyubiquitination that will mark the target protein for proteosomal degradation^{37, 38, 39, 40}.

Some of the components of the destruction complex

The structures of some of the components of the destruction complex will only be described briefly.

The scaffolding protein **Axin** has the lowest abundance in the cell, and is therefore probably the limiting protein in the complex^{41, 42}.

APC is a large protein. Three areas of the protein are especially important in this context; APC is connected to Axin through three series of SAMP (Serine-Alanine-Methionine-Proline), and β -catenin is bound to APC through three series of a 15 amino acid (aa) sequence and seven 20 aa repeats³³. APC gets phosphorylated by GSK3 and CK1 ϵ , and this increases the affinity for β -catenin³³.

CK1 is a family of kinases where several of the members have an effect on the canonical Wnt pathway, both with and without Wnt. CK1 ϵ is already mentioned to phosphorylate APC. CK1 α bound to Axin phosphorylates β -catenin, and primes β -catenin for further phosphorylation by GSK3⁴³. CK1 α phosphorylates Ser45⁴⁴, while GSK3 phosphorylates at least Ser33, Ser37 and Thr41 at the N-terminus⁴⁵.

At the C-terminus in **GSK3** a hydrophobic groove connects to Axin, and the active site of GSK3 may then phosphorylate β -catenin at the N-terminus, which makes a docking site for the E3 ubiquitin ligase³³.

In the nucleus

Groucho is a non-DNA binding co-repressor⁴⁶ that binds to TCF/LEF together with HDAC (histone deacetylase) and inhibits transcription of genes^{47, 48}.

1.3.3 The canonical Wnt signaling pathway – ON-State

The Wnt proteins are the ligands that turn this signaling pathway on²⁸. They are secreted signaling molecules with a huge variety of functional properties, and are therefore defined by their amino acid sequence⁴⁹. They have a similarity of at least 18 %⁵⁰. By October 2009 there were 19 described human Wnt genes with similarity between 27 % and 83 %, coding for proteins of 350 to 400 amino acids^{49, 51}. All of them have several highly charged amino acid residues, and they all also have 23-24 conserved cysteine residues⁴⁹. Wodarz & Nusse have suggested that Wnts may be divided into two classes, the XWnt-8 class that may lead to axis duplication in *Xenopus*, and the XWnt-5A class that does not lead to axis duplication⁵². This is consistent with the classification into a canonical Wnt1-class and a non canonical Wnt5a-class.

After the Wnt-proteins are secreted from the cell they may interact both with the cell they were secreted from, neighbor cells, and cells that are up to 20-30 cell diameters distant from the secreting cell^{53, 54}.

At the cell surface

At the cell surface Wnt-proteins may interact with a family of serpentine receptors called Frizzled (Frz), and a family of co-receptors called low-density lipoprotein receptor-related proteins (LRP)^{55, 56}.

Frz is a family of transmembrane proteins that cross the cell membrane seven times^{57, 58, 59}. It has an amino-terminal extension that contains a signal sequence, and a highly conserved cysteine-rich domain (CRD) of 10 amino acids (aa) that Wnt may bind to outside the cell^{57, 58, 59}. A linker containing 40-100 aa that is different from protein to protein binds the CRD to a transmembrane segment^{57, 58, 59}. Loops join the seven transmembrane segments, and a tail inside the cell may transfer the signal further^{57, 58, 59}.

LRP is also a transmembrane protein, but it only crosses the cell membrane once. It has a large domain outside the cell, and a smaller domain in the cytosol. It has been suggested that LRP changes conformation when Wnt binds to it, and that this leads to binding to Frz^{58, 59}.

In the cytoplasm

Dishevelled (Dvl) is a phosphoprotein that can interact with several signal components. It is built up of three highly conserved domains; DIX, PDZ, and DEP. At the amino-terminal end there is a 50 aa domain called DIX, which has its name from Dvl and Axin because it has an area that is similar to Axin^{60, 61}. The central region contains a PDZ domain with 80-90 amino acids⁶². This domain has its name from the three first proteins that were discovered to contain PDZ; Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA) and zonula occludens-1 protein (zo-1). At the carboxy-terminal there is a DEP domain. The DEP-domain has its name from Dvl, Egl-10, and Pleckstrin.

In the presence of Wnt binding, the scaffolding protein Dvl is phosphorylated on serine and threonine residues by several kinases, among others **casein kinase 2 (CK2)**⁶³.

Dvl will then be attached to the cell membrane, and will here help LRP aggregate and be phosphorylated by **CK1 γ** ⁶⁴ at T1479 in the intracellular domain of LRP⁶⁴, in addition to phosphorylation by **GSK3 β** . The phosphorylation of LRP will give Axin a higher affinity for LRP than for the proteins in the destruction complex, and the destruction complex will not be formed⁶⁵.

β -catenin will not be ubiquitinated and degraded to the same extent, and its concentration in the cytoplasm will increase. Some of the β -catenin will translocate to the nucleus⁶⁶.

In the nucleus

After the β -catenin enters the nucleus it functions as a temporary framework to bind T-cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) to the Wnt target genes and trigger a transcription of these genes⁶⁷. A list of 117 target genes and 14 feedback target genes can be found at The Wnt Homepage^{68, 69}.

1.4 Small molecules in inhibition or activation of the canonical Wnt signaling pathway

An inappropriately activated canonical Wnt signaling pathway could lead to a range of human diseases and numerous cancers. Small molecules that will modulate the canonical Wnt signaling pathway could therefore prove to be important in the making of pharmacological agents. Several screenings have already been performed to find small molecules that affect the signaling pathway at different levels^{71, 72, 73, 74, 75}.

Lithium chloride is one of the most effective chemical treatments for bipolar disorder. Klein and Melton⁷⁰ found that lithium may inhibit GSK3, which is one of the components in the canonical Wnt signaling pathway.

Zhang et al⁷¹ screened a library of 100.000 small molecules to find a molecule that synergize with Wnt-3A. They found a purine derivative, QS11, which bound to a GTPase (ARFGAP1) and inhibited its function. Figure 3 shows the identified small molecule.

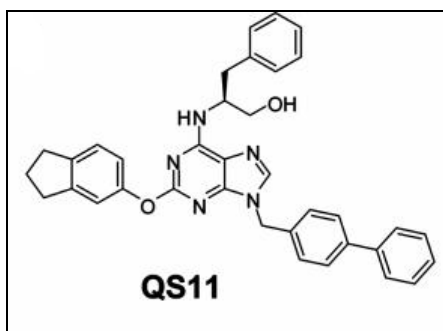


Figure 3 Structure of QS11⁷¹.

Bodine et al⁷² screened 440.000 drug-like compounds for inhibitors of secreted frizzled-related protein 1 (sFRP-1), and found a diphenylsulfone sulphonamide that inhibited human sFRP-1 in U2-OS cells. sFRP-1 is a Wnt antagonist, and inhibition of this protein will lead to activation of the canonical Wnt signaling pathway.

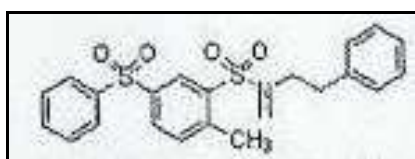


Figure 4 Structure of a diphenylsulfone sulphonamide that inhibit human sFRP-1⁷².

Huang et al⁷³ identified a small molecule that could prolong the halflife of Axin and promote β -catenin degradation through inhibiting tankyrase (TNKS). Figure 5 shows the structure of the identified small molecule.

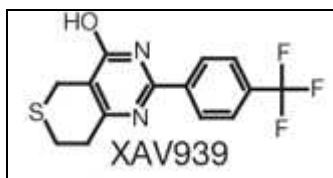


Figure 5 Structure of XAV939⁷³.

Zhong et al⁷⁴ screened a chemical library of 4000 compounds and identified a GSK3 inhibitor (Figure 6).

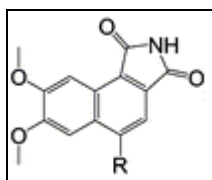


Figure 6 Structure of GSK3 inhibitor⁷⁴.

Emami et al⁷⁵ screened a library of 5000 small molecule compounds, and found a compound that inhibited the canonical Wnt signaling pathway by binding to cyclic AMP response element-binding protein (CBP). CBP is one of the transcriptional coactivators of β -catenin. Figure 7 shows the structure of the CBP binding compound.

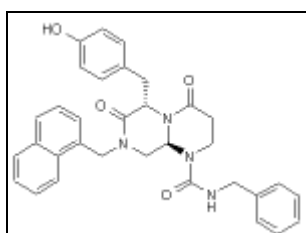


Figure 7 Structure of CBP binding compound⁷⁵.

These examples show some of the types of small molecules that affect the different parts of the canonical Wnt signaling pathway. Most of them contain aromatic rings, some contain lactam and cyclic amine, but a structure as simple as LiCl also affects this signaling pathway.

1.5 The Sonic hedgehog signaling pathway

The hedgehog signaling pathways is largely involved in embryo development and cell division of adult stem cells in a variety of species.

In mammalian species the hedgehog-family of ligands are called Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh).

Figure 8 shows the main steps, and a very brief description of the hedgehog signaling pathway in vertebrates follows^{76, 77}.

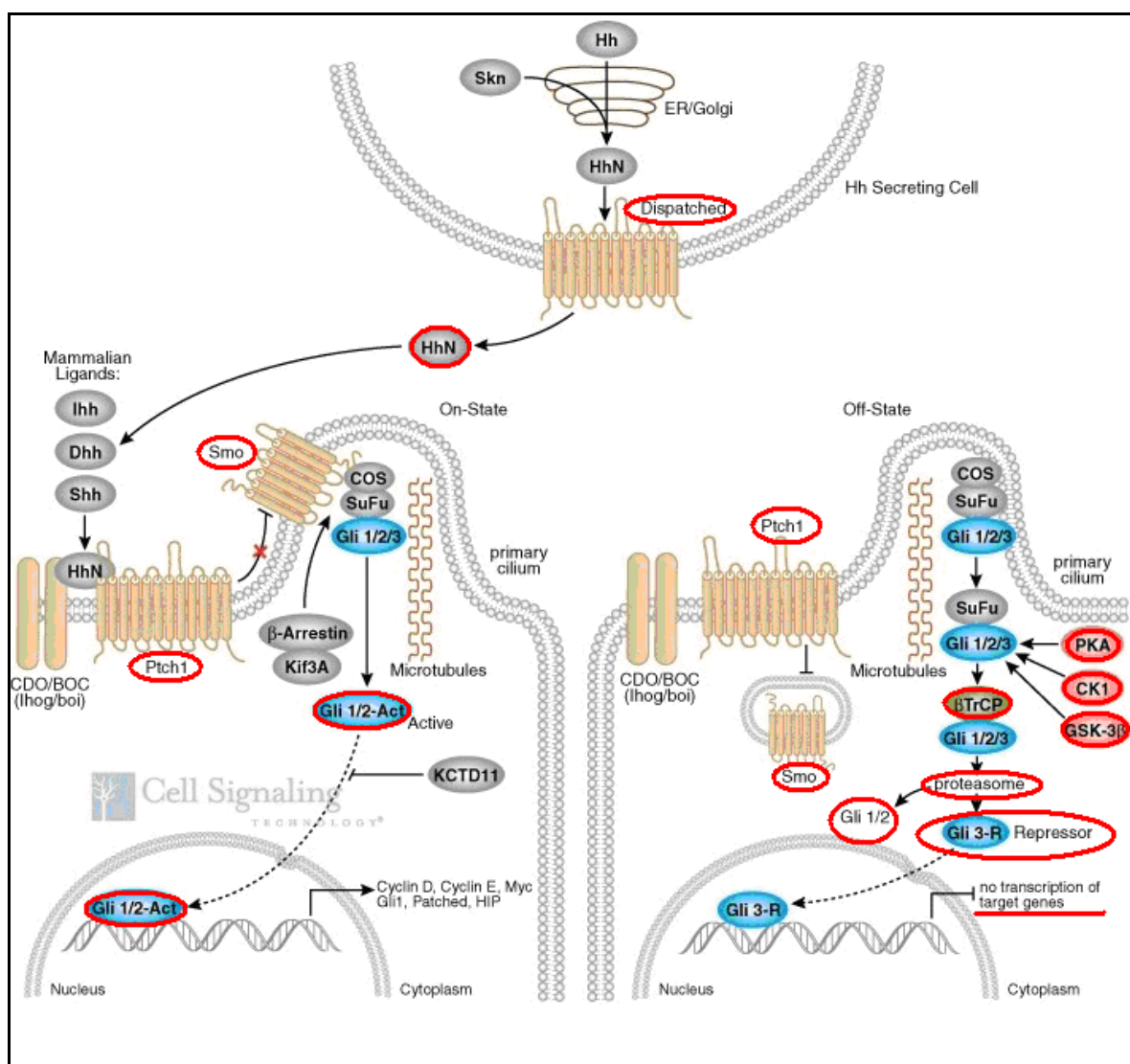


Figure 8 Simplified illustration from Cell Signaling Technology⁷⁷ of the hedgehog signaling pathway in vertebrates.

OFF-state

Receptors at the cell surface, called Patched (Ptc), act as suppressors on a protein called Smoothed (Smo) in the absence of the hedgehog ligand, and prevents this protein from translocating to the primary cilium.

A transcription factor called Gli will be phosphorylated by PKA, CK1 and GSK3, and a β -TrCP-mediated degradation of Gli activators or generation of repressor-Gli will follow. This will lead to repression of hedgehog target genes.

ON-state

The hedgehog ligands go through multiple processing steps before a dually lipid-modified hedgehog ligand with a N-terminal signaling domain will be secreted from Dispatched in the secreting cell. The hedgehog ligands will be transported over both short and long distances in the body, and will eventually bind to Ptc. This will translocate Smo to the primary cilium. Gli translocates to the nucleus and activate hedgehog target genes.

1.6 The NF- κ B (nuclear factor kappa B) signaling pathway

The NF- κ B signaling pathway plays a huge role in immune- and inflammatory responses. This signaling pathway can be activated in a wide range of ways, stimulation by the proinflammatory cytokine tumor necrosis factor α (TNF- α) being one of them.

Figure 9 shows the main steps in this very complex signaling pathway, and a very brief description of the NF- κ B signaling pathway activated with TNF- α follows⁷⁸.

OFF-state

A family of inhibitors, called I κ Bs (inhibitors of κ B), bind to NF- κ B in the cytoplasm and prevent it from entering the nucleus.

ON-state

TNF- α bind to its receptor at the cell surface, and this will finally lead to phosphorylation of the I κ Bs. β -TrCP will then mark the I κ Bs for ubiquitination and proteosomal degradation. The transcription factor NF- κ B will be translocated into the nucleus and bind the DNA at κ B binding motifs.

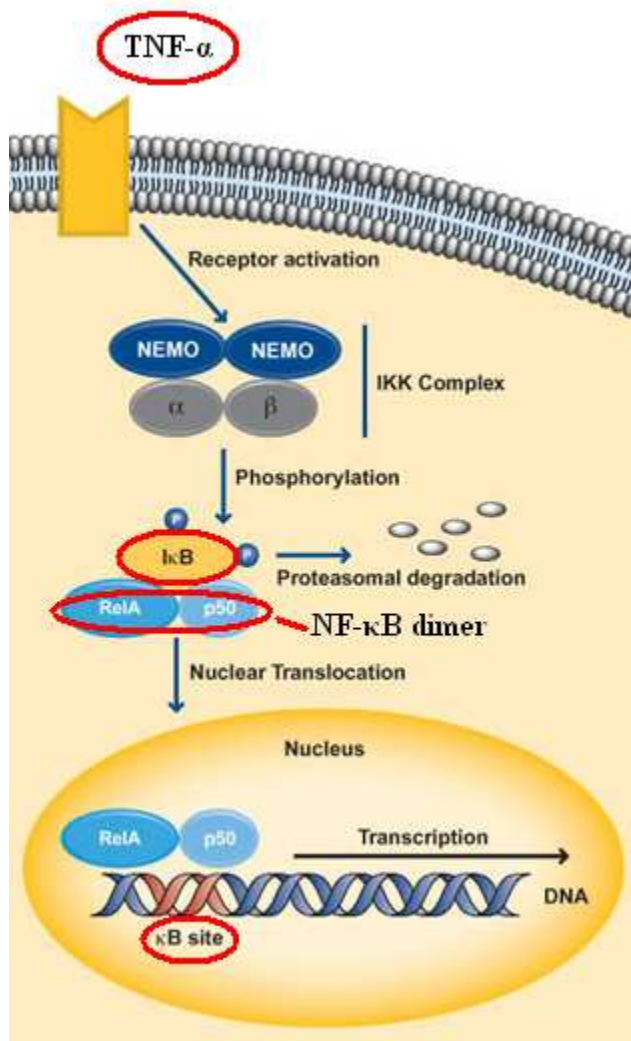


Figure 9 Simplified illustration from abcam⁷⁸ of the NF-κB signaling pathway.

1.7 Cells and methods

1.7.1 Cells and conditioned medium

Human embryonic kidney 293 wild type (HEK293 wt) cells is a cell line generated from a healthy aborted fetus in early 1970s, and the cell line was first described in 1977⁷⁹. This cell line is widely used in cell biology research because it is easy to grow and transfect very readily. For our purpose it is suitable because it contains the components of the canonical Wnt signaling pathway, except Wnt itself. HEK293 are not Wnt producing cells. These cells also contain the components of the NF- κ B signaling pathway but are not NF- κ B producing cells.

HEK293 Super TOP-Flash (STF) cells contain the integrated variant of the Wnt/ β -catenin-responsive luciferase reporter Super TOP-Flash, which gives us an excellent way to measure the response to activation by Wnt. This cell line is clonally selected for high sensitivity (strong signal) for the canonical Wnt signaling pathway. (For more information about luciferase assay and STF see section 1.7.4 and 1.7.5)

Sonic hedgehog (Shh)-Light 2 cells are derived from the NIH/3T3 cell line (mouse embryonic fibroblast cells)⁸⁰ and contain the integrated variant of the GLI-responsive firefly luciferase reporter and a *Renilla* luciferase expression vector. This cell line contains the components of the Shh signaling pathway. (For more information about luciferase assay, GLI-responsive firefly luciferase and *Renilla* luciferase see section 1.7.4 and 1.7.5).

Conditioned medium is per definition medium collected from a growing cell line which secret biologically active components, and these components will affect some cell functions (e.g. signaling pathways)⁸¹.

To activate the canonical Wnt signaling pathway, Wnt-proteins have to be added. Murine L1 cells stably transfected with a Wnt-3A expression vector⁸² produce and secrete Wnt-3A proteins, which are known to activate the canonical Wnt signaling pathway, and Wnt-3A conditioned medium (also called L1-medium) can be obtained from these cells.

Conditioned Shh-medium is collected from PANC-1 cells (human pancreatic carcinoma, epithelial-like cell line) stably transfected with a Shh expression vector.

1.7.2 Coating well plates with poly-L-lysine

When well plates are coated with poly-L-lysine hydrobromide (molecular formula: $(C_6H_{12}N_2O_2)_n \cdot xHBr$) the adhesion increases and the chance of cell loss will be reduced.

Poly-L-lysine improves electrostatic interactions, by increasing the number of positively charged ions on the well surface so the negatively charged ions of the cell membrane will have more binding sites⁸³. Figure 10 shows the chemical structure of poly-L-lysine hydrobromide.

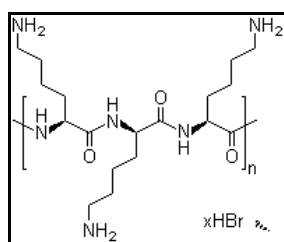


Figure 10 Chemical structure of poly-L-lysine hydrobromide from chemBlink⁸⁴.

1.7.3 Transfection

Transfection is the process by which exogenous DNA or RNA is introduced into cells⁸⁵. A vector is in molecular biology and genetics defined as a DNA molecule used to transfer this exogenous DNA or RNA into the cell^{86, 87}.

DNA can be transfected into the cell as a plasmid (a type of vector). A plasmid is per definition a circular molecule of double stranded DNA that can be replicated inside the cell (independent of the chromosomal DNA)⁸⁸. The plasmid DNA can be stably transfected into the cells genome or transiently transfected into the cell⁸⁹. With the DNA transiently transfected, it will be degraded and diluted through mitosis and the expression will only occur in a short period of time⁹⁰. By chance some of the plasmid DNA will be included into the genome, and by adding a selection marker (gene for drug resistance) into the plasmid only the cells with the plasmid integrated into the genome will survive when treated with the selection chemical⁹¹. This selection will give a cell line with the plasmid stably integrated into the cells genome, and the genes from the plasmid will be replicated along with the rest of the DNA through mitosis.

Two stably transfected cell lines were used in this master thesis; HEK293 STF cells and Shh-Light2 cells. Transiently transfection was used on HEK293 wt cells with a combination of one reporter plasmid and one constitutive plasmid (STF plasmid as a reporter for the canonical Wnt signaling pathway and NF- κ B-luc plasmid as a reporter for the NF- κ B signaling pathway, and a *Renilla* plasmid as a constitutive plasmid for internal control. See section 1.7.4 and 1.7.5).

Several methods can be used to transfect DNA into cells, but only chemical-based transient transfection with FuGENE-6™ has been used in this master thesis. FuGENE-6™ is a cationic lipid that will bind to the negatively charged DNA. Endocytosis will then transport the DNA/ FuGENE-6™ complex into the cell⁹².

1.7.4 Luciferase assay

Activation of the canonical Wnt signaling pathway will lead to transcription of a range of genes, and to quantify the activation of the signaling pathway the gene transcription needs to be quantified. Luciferase assay is a method used to relate luciferase-mediated bioluminescence to the activation of a signaling pathway such as the canonical Wnt signaling pathway, or other cellular events coupled to gene expression.

A type of enzyme, called luciferase, catalyzes the oxidation of the organic molecule luciferin, and this reaction leads to emission of light that is proportional to the amount of luciferase in the reaction⁹³.

Several different types of luciferase can be used, and in this master thesis the *Photinus pyralis* firefly luciferase and the *Renilla reniformis* *Renilla* luciferase were employed.

The enzyme *Photinus pyralis* firefly luciferase catalyzes oxidation of *Photinus* luciferin while *Renilla reniformis* *Renilla* luciferase catalyzes oxidation of *Renilla* luciferin (see Figure 11)^{93, 94, 95, 96, 97, 98}.

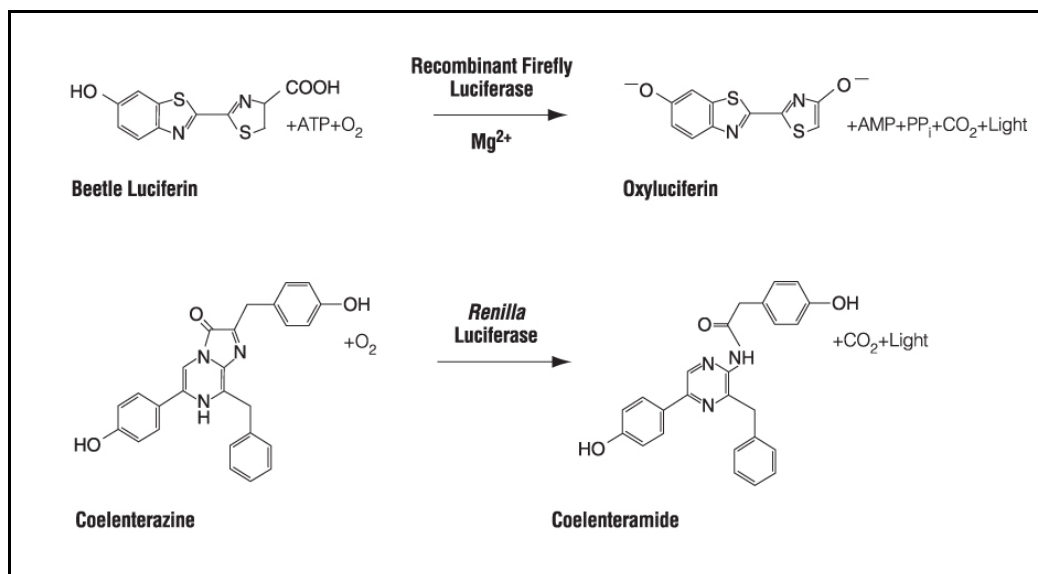


Figure 11 The oxidation reactions used in a Dual-Luciferase Reporter Assay (coelenterazine is a synthetic *Renilla* luciferin)⁹⁸.

Both firefly luciferase and *Renilla* luciferase can be used as genetic reporters to measure the transcription of other genes^{94, 97}. This is done by attaching the reporter gene (luciferase) to the regulatory sequence of the gene that will be transcribed when e.g. the canonical Wnt signaling pathway is activated. When this other gene is targeted for transcription, the luciferase gene will also be transcribed.

In this master thesis only the firefly luciferase was used as a reporter gene, while the *Renilla* luciferase was used as a constitutive active gene in some of the experiments to provide an internal control of cell viability and transfection rate (Dual-Luciferase Assay System)⁹⁸.

The genes coding for the enzymes are placed inside the cells (stably transfected into the genome of a cell line or transiently transfected, see section 1.7.3), and the constitutive gene will be transcribed all the time while the reporter gene will be transcribed when the signaling pathway is activated. To measure the transcription of the genes, luciferin is added before measuring the luminescence with a luminometer. With a Dual-Luciferase Assay System Beetle Luciferin is added and the luminescence is measured, then this reaction is stopped and Coelenterazine is added, and the luminescence is measured again (see section 2.7).

In this master thesis, the luciferase assay has been used with:

- 1) HEK293 STF cells: a stably transfected cell line with only canonical Wnt-responsive firefly luciferase.
- 2) Shh-Light2 cells: a stably transfected cell line with both firefly luciferase (GLI-responsive) and *Renilla* luciferase.
- 3) Transient transfection with both firefly luciferase and *Renilla* luciferase on HEK293 wt cells. NF- κ B-responsive firefly luciferase (NF- κ B-luc plasmid) for the NF- κ B signaling pathway, and canonical Wnt-responsive firefly luciferase (STF plasmid) for the canonical Wnt signaling pathway.

1.7.5 Plasmids

A plasmid is as mentioned in section 1.7.3 a type of vector. A plasmid generally contains a promoter, the gene(s) to be transcribed, and a polyA tail. The promoter facilitates the transcription and can be constitutive (continuous transcription) or inducible (induced by the presence or absence of other factors). The polyA tail terminates the transcription and adds adenosine residues to increase the stability and translation of the RNA transcript.

In addition a gene that confers antibiotic resistance and an origin of replication (where the replication is initiated) is often present in the plasmid.

The *Renilla* plasmid pRL-TK was used in this master thesis as an internal control for the transfection and viability of the cells. The pRL-TK Vector circle map from Promega is shown in Figure 12.

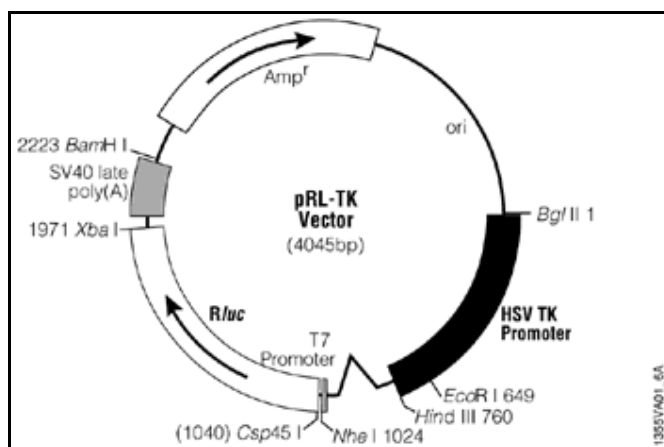


Figure 12 The pRL-TK Vector circle map and sequence reference points⁹⁹. A HSV-TK (herpes simplex virus thymidine kinase) promoter ensure constitutive expression of the *Renilla* luciferase reporter gene (*Rluc*), and the SV40 late poly(A) add 200-250 adenosine residues to the transcript.

As the reporter vector for the canonical Wnt signaling pathway a construct called Super8xTOPFlash (made by Ajamete Kaykas in the Moon lab) was used in this thesis. The vector circle map is shown in Figure 13. The cell line HEK293 STF has this plasmid stably transfected into its genome.

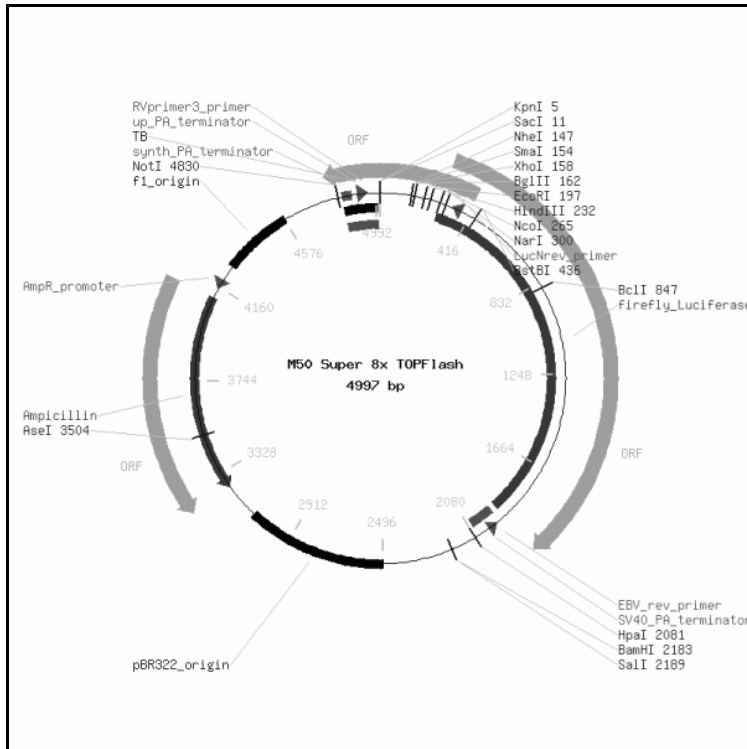


Figure 13 The Super8xTOPFlash Vector circle map and sequence reference points¹⁰⁰. This construct is made up of a pTA-Luc vector backbone (which drives the expression of the firefly luciferase) from Clontech and with 7 TCF/LEF binding sites incorporated¹⁰⁰ to get the firefly luciferase gene transcribed when the canonical Wnt signaling pathway is activated.

As the reporter vector for the NF- κ B signaling pathway a construct called NF- κ B-Luc was used in this master thesis. This reporter vector contains NF- κ B response element (κ B₄) bound to a TATA-like promoter (P_{TAL}). When NF- κ B is translocated into the nucleus it will bind to κ B₄ and induce transcription of the reporter gene firefly luciferase (luciferase). A SV40 polyA add adenosine residues to the transcript.

With the Shh signaling pathway a stably transfected cell line (Shh-Light2) derived from the NIH/3T3 cell line (mouse embryonic fibroblast cells)⁸⁰ and transfected with GLI-responsive firefly luciferase reporter and a *Renilla* luciferase pRL-TK.

1.7.6 Real-Time reverse transcription-polymerase chain reaction

Polymerase chain reaction (PCR) is a technique used to generate many copies of a specific DNA sequence, invented already in 1971¹⁰¹. Reverse transcription-PCR (RT-PCR) is a variant of PCR where an RNA strand is reverse transcribed into its complementary DNA (cDNA) by using the enzyme reverse transcriptase. This cDNA is then amplified using PCR. Real-time RT-PCR is used to monitor the advancement of a PCR reaction in real time, and to quantify the targeted DNA molecule¹⁰².

The mixture for the real-time RT-PCR needs to contain cDNA, DNA polymerase, and primers. Before elaborating on the DNA polymerase and the primers, the obtaining of the cDNA will be explained.

24 hours after treating HEK293 wt cells with compounds and conditioned medium, the RNA from the cells are released by cell lysis. Mercaptoethanol is added to inhibit formation of ribonucleases (RNases, which catalyzes the degradation of RNA), by breaking disulfide bonds¹⁰³. The RNA is purified using manufacturers procedure.

After the mRNA concentration is measured according to their optical density with a spectrophotometer, cDNA is synthesized from the mRNA by using an enzyme called reverse transcriptase. Dithiothreitol (DTT) is added to inhibit RNases. The mixture also contains deoxynucleoside triphosphates (dNTP) which are the building blocks for DNA strands, and a random hexamer primer that binds at non-specific point along the RNA template to synthesize all the RNA to cDNA.

The produced cDNA is mixed with the SYBR® Green PCR Master Mix, which contains a dye called SYBR Green 1 which is used to detect and quantify the gene transcripts. This dye binds non-specifically to double-stranded DNA, and has an excitation maximum at 497 nm and an emission maximum at 520 nm¹⁰⁴. The SYBR® Green PCR Master Mix also contains AmpliTaq Gold® DNA Polymerase and dNTPs. A passive reference dye to normalize pipetting volumes is also added, and the one most commonly used is ROX™ Passive Reference Dye. The primers to be used are also added to this mix. A primer is in molecular biology and genetics defined as a strand of nucleic acid (DNA or RNA) that is complementary to a given DNA sequence and serves as a starting point for DNA replication by the enzyme DNA polymerase^{105, 106}. One set of primers (forward and reverse) is needed for each cDNA sequence we want to replicate. Glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) is often used as an internal reference gene to normalize the expression levels¹⁰⁷.

The PCR-method is based on thermal cycling, but before the cycles starts the mixture is heated up to 94-96 °C for 1-9 minutes to denaturize all the DNA. The thermal cycling consists of three steps which are repeated 20-40 times. The first step is to denature the DNA, which is done by heating the mixture to 94-96 °C, but only for 20-30 seconds. The next step is to help the primers anneal to the single stranded DNA, by lowering the temperature to 50-65 °C (dependent on the primers used) for 20-40 seconds. In the last step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand. The temperature in this step is dependent on the DNA polymerase used (as an example Taq polymerase needs 75-80 °C to function optimal). After the cycles are finished, a final elongation is performed at 70-74 °C for 1 to 15 minutes to make sure the entire single-stranded DNA is extended.

Software can be used to perform automated gene expression analysis to quantify and normalize the results from the Real-Time RT-PCR.

1.8 The aim of this master thesis

The canonical Wnt signaling pathway controls embryo development and adult stem cell maintenance. When incorrectly activated, the canonical Wnt signaling pathway may lead to a range of human diseases, including cancers^{16, 22, 23}, Alzheimers¹⁷, multiple sclerosis¹⁸ and arthritis²¹. To have the power to control the canonical Wnt signaling pathway and correct improperly activation could possibly treat or prevent these diseases. As mentioned in section 1.4, LiCl is used to treat bipolar disorder (lithium inhibits GSK3 beta, which is one of the main players in the canonical Wnt signaling pathway).

The importance of adjusting this signaling pathway led to the search for small molecules that influence different steps in the canonical Wnt signaling pathway. Several small molecules other than LiCl have been found to have an effect (see section 1.4).

In addition to the search for pharmacological agents, the small molecules may also be used as scientific experimental tools for examination of other components of the signaling pathway, both in vivo and in vitro.

In this master thesis the binding between the Wnt ligand and the Frizzled receptor has been in focus. Finding a small molecule that increased or decreased the binding between Frz and Wnt giving a synergistic or inhibitory effect, was the aim of the study.

A computer based (*in silico*) screening of 10.000 compounds done by Andrew Voronkov prior to the study (see section 2.9) limited the laboratory screening to 256 compounds.

From these 256 small molecules the best inhibitors and/or the best synergists were to be found. Analogues of the most promising compounds from the screening were also going to be examined to see if even better inhibitors and/or synergists could be found.

2 Experimental – kits, materials and methods

2.1 Cell culture and plating of cells

Adherent cell lines were used, and grown in 25 cm², 75 cm² and 175 cm² sterile Nalge Nunc EasyFlask Culture flasks (NUNC – Rochester, NY, USA).

HEK293 STF cells, kindly provided by prof. dr. Vladimir Korinek (IMG, ASCR, Prague, Czech Republic), HEK293 wt cells (ATCC – Manassas, VA, USA), murine L1 cells stably transfected with Wnt-3A expression vector (ATCC), and Shh-Light2 cells (ATCC) were all sub cultured two or three times a week in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-1 (Invitrogen™ - Carlsbad, CA, USA) supplemented with 10% (v/v) inactivated Foetal Bovine Serum (FBS) (Invitrogen™) and 1% (v/v) Penicillin streptomycin (Pen Strep, PS) (BioWhittaker® – Walkersville, MO, USA) (abbreviated DMEM/FBS/PS, or referred to as regular cell medium from now on). HEK293 STF, L1 and Shh-Light2 cells were supplemented with 0.25 µg/µL Geneticin G418 (Invitrogen™), and Shh-Light 2 cells were also supplemented with 0.25 µg/µL Zeocin (Invitrogen™).

Phosphate buffered saline, PBS (Rikshospitalet, Norway) (see Attachment 1: Recipes), was used to wash away rests of medium, before trypsination with Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) (BioWhittaker®) to loosen the cells from the surface inside the growth flask.

A counting chamber (Bürker, Germany) was used to determine the concentration of cells¹⁰⁸.

To do the experiments, the cells were evenly distributed in wells in a well-plate (wp) (NUNC). For HEK293 wt and HEK293 STF cells, plates coated with poly-L-lysine were used. 80k cells per well (c/w) was added to each well to a final volume of 500 µL in a 48 wp, and 320k c/w to each well to a final volume of 2 mL in a 12 wp. For Shh-Light2 cells 100k c/w was added to each well in a 48wp. DMEM/FBS/PS-medium was used for all the plated cells, and the cells were grown overnight before adding compounds.

L1 cells were used to make Wnt-3A conditioned medium (2.2.1).

2.2 *Production of conditioned medium and other medium*

2.2.1 L1-medium

3 mL Trypsin-EDTA was added to the 75 cm² cell flask with L1-cells of approximately 100% confluence, after removing the medium and washing with PBS. After 5 minutes incubation the mixture was gently homogenized and 0.66 mL were transferred to a 175 cm² cell flask. 31.25 mL DMEM/FBS/PS were added. After 72 hours the medium was carefully removed from the flask without detaching the cells. 31.25 mL new medium (DMEM/FBS/PS) was added to the flask. The old medium was sterile filtrated through Whatman® 0.2 µm filter (GE Healthcare, England) and stored at 4°C (M1). After 96 hours, the medium on the cells was carefully removed from the flask, and the flask was thrown away. The collected medium was sterile filtrated (M2) and combined with M1.

2.2.2 Shh-medium

Conditioned Shh-medium was kindly provided by Martin Strand (Cellular and Genetic Therapy Research Group, Institute of Microbiology, Rikshospitalet, Norway) from Shh-PANC-1 (Human pancreatic carcinoma, epithelial-like cell line) cells stably transduced with a mouse Shh-expression vector.

2.2.3 Medium with TNF-α

Recombinant rat TNF-α (R&D Systems – Minneapolis, MN, USA) was dissolved in DMEM/FBS/PS to a final concentration of 10 ng/mL.

2.3 *Poly-L-lysine; preparing stock solution and coating plates*

A 5 mg/mL stock solution was prepared by solving 100 mg poly-L-lysine hydrobromide (15-30k mol.mass) (Sigma-Aldrich – St.Louis, MO, USA) in 20 mL Milli-Q (MQ) H₂O (Milli-Q® systems, Millipore – Billerica, MA, USA), aliquoted into 1.5 mL eppendorf tubes (VWR – West Chester, PA, USA) and stored at -20°C.

For coating of the plates a solution of 70 µg/mL was used, made by appropriate dilution of the stock solution. The solution was added to the wells, left for 2 hours and then the wells was washed twice with autoclaved MQ-H₂O. The wells were dried before use.

2.4 Adding compounds to investigate their effect on endogenous luciferase activity the Wnt/ β -catenin signaling pathway

HEK293 STF cells were plated in a 48 wp coated with poly-L-lysine and grown overnight. Each compound dissolved in dimethyl sulfoxide (DMSO) (Promega) to be investigated was added to an Eppendorf tube (Eppendorf HQ – Hamburg, Germany), and then mixed with conditioned Wnt-3A medium or with DMEM/FBS/PS. The mixture was vortexed and carefully added to the wells without detaching the cells. DMSO was added to the negative and positive controls because the compounds were dissolved in DMSO. After approximately 24 hours the Luciferase activities were measured (2.7).

2.5 Adding compounds to investigate their effect on the Shh-signaling pathway

Shh-Light2 cells were plated in a 48 wp and grown overnight. A compound dissolved in DMSO was added to a tube, and then mixed with conditioned Shh medium. The resulting mixture was vortexed and carefully added to a well without detaching the cells. DMSO was added to the negative and positive controls. After approximately 48 hours the Luciferase activities were measured (2.7).

2.6 Transfection with FuGENE® 6

HEK293 wt cells were plated in a 48 wp coated with poly-L-lysine and grown overnight. The transfection was performed with FuGENE® 6 Transfection Reagent (Roche – Basel, Switzerland), using standard protocol from Roche (Version February 2006) with a ratio 3:1 of FuGENE® 6 (μL) : plasmid (μg) (0.75 μL FuGENE® 6, 0.25 μg plasmid). OPTI-MEM® I Reduced Serum Media (modification of MEM)(Invitrogen™) was used.

A mixture of two plasmids constituted the 0.25 μg of plasmid in the ratio, and the types of plasmids depended on the signaling pathway under investigation.

To investigate the canonical Wnt signaling pathway, a Super TOP-Flash plasmid (pSTF) (Promega) with a stock solution of 2.265 μg/μL, and a *Renilla* plasmid (pRL-TK) (Promega) with stock solution 10 ng/mL were used.

To investigate the NF-κB signaling pathway, a pNF-κB-luc (Panomics – Fremont, CA, USA) with a stock solution of 1 μg/μL was used together with the *Renilla* plasmid.

In a 48 wp each well was added 20 ng of the *Renilla* plasmid and 230 ng of the STF plasmid or the NF-κB plasmid.

18 hours after transfection the compound (dissolved in DMSO) to be investigated was added to a tube, and then mixed with either conditioned Wnt-3A medium (canonical Wnt signaling pathway and pSTF), or 10 ng/mL TNF-α-medium (NF-κB signaling pathway and pNF-κB). The mixture was then vortexed and carefully added to a well without detaching the cells. DMSO (Promega) was added to the negative and positive controls. After approximately 24 hours the Luciferase activities were measured (2.7).

2.7 Luciferase activity measurement

Luciferase activity measurement was performed with either a Dual-Luciferase® Reporter Assay System (Promega – Sunnyvale, CA, USA) or with a Luciferase Assay System (Promega), and with standard protocols from Promega (Part# TM040 and Part# TB281). The activities were measured with a 20/20ⁿ Luminometer (Turner BioSystems/Promega).

2.8 Real time RT-PCR

HEK293 wt cells were grown in a 12 wp, 320 k c/w. Extraction of RNA from cells was performed using a Genelute Mammalian total RNA kit (Sigma), and a standard protocol (RTN 350, 70, 10).

The mRNA concentrations were measured by adding 200 μ L Tris-EDTA (TE) buffer (Rikshospitalet, Norway) (see Attachment 1: Recipes) to 2 μ L mRNA solution. After pipette mixing, vortex mixing and a rapid centrifugation for 2-5 seconds the mRNA concentration was measured according to their optical density in an Ultrospec™ 2100 pro UV/Visible Spectrophotometer (GE Healthcare).

The cDNA synthesis was performed using AffinityScript™ Multiple Temperature Reverse Transcriptase (Stratagene – La Jolla, CA, USA) and a standard protocol (Catalog #200436). Brilliant® SYBR® Green QPCR Master Mix (Stratagene) with standard protocol (Catalog #600548) was used to make the PCR reaction mixture.

The primers used were:

Axin2 forward: 5'-CCCAAGCCCCATAGTGCCCAAAG-3'

Axin2 reverse: 5'-CAGGGGAGGCATCGCAGGGTC-3'

GAPDH forward: 5'-GCCCCCTCTGCTGATGCCCCCA-3'

GAPDH reverse: 5'-TGGGTGGCAGTGGCATGG-3'

The Stratagene Mx3000P QPCR System (SuperArray Bioscience Corporation – Frederick, MD, USA) was used for the Real Time RT-PCR. (Initial denaturation at 95°C for 5 minutes. 40 cycles of denaturation, annealing and amplification using 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. Ending performed at 95°C for 60 seconds.)

MxPRO™ QPCR Software (Stratagene) was used to perform automated gene expression analysis to quantify and normalize Axin2 against GAPDH.

2.9 Compounds

Totally 482 compounds (C1089-C1344, C1355-C1580) were provided by ChemDiv (San Diego, CA, USA). The structures may not be revealed due to secrecy until publication by the Section for Cellular and Genetic Therapy, Institute of Microbiology, Rikshospitalet, Norway. The compounds were dissolved in DMSO to a concentration of 10 mM and kept at 4 °C until use.

Selection of compounds was carried out by Andrew Voronkov (Moscow State University, Department of Chemistry), and the following page is written by him:

As initial protein structure of CRD domains the structure 1ijy from protein databank (RCSB.org) was used (Ref. 1). This structure corresponds to CRD domain of Frizzled 8 mice receptors. The water molecules were removed from the 1ijy structure, which then was used to construct homology based models of CRD domains for other human Frizzled receptors. After homology based modelling the structures quality was evaluated by ProCheck module of Sybyl7.1 software (Ref. 2). The hydrogens and Kollman all charges were added to protein structures. The protein geometry was then optimized by molecular mechanics methods using Tripos force field (Ref.3).

Initial database of 10 000 compounds from ChemDiv diversity set was used for virtual screening procedure through each of dimeric CRD domains of Frizzled receptors. Virtual screening procedure was performed using Gold3.1 software at Moscow State University. The binding sites on CRD domains interface were chosen (sites 2, 3, 4 on the Fig.1). The binding sites were selected based on protein-protein docking studies of Wnt protein with dimeric CRD domain and according the experimental data on point mutagenesis (Fig.2). The compounds for the next receptors were selected: Frizzled 6, Frizzled 7, Frizzled 8 as dataset of 250 compounds after screening. These compounds were sent for experimental trials to Professor Stefan Krauss group.

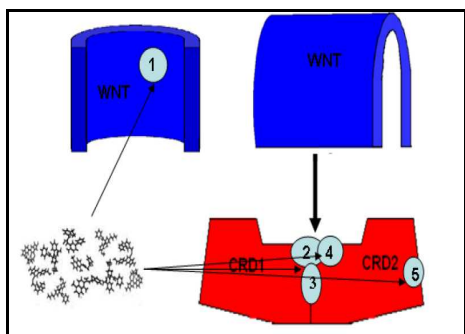


Fig. 1 Potential small molecule action sites on the surface of Wnt protein and dimeric CRD domains of Frizzled receptors

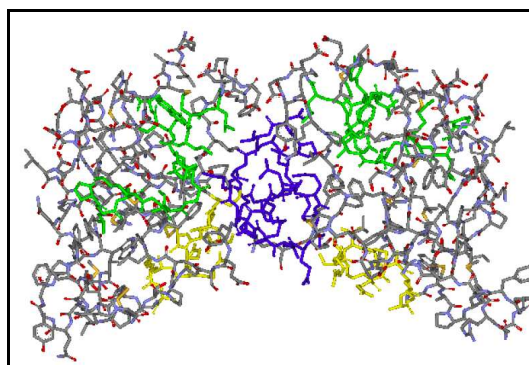


Fig. 2 Point mutagenesis data visualised on dimeric CRD domain. Blue – amino acids which are supposed to be important for CRD domain dimerization. Green – amino acids which are supposed to be important for Wnt binding. Yellow - amino acids which are supposed to be responsible for correct orientation against transmembrane part of Frizzleds.

References:

1. Dann C. E., Hsieh J. C. , Rattner A. , Sharma D., Nathans J., Leahy D. J., Insights into Wnt binding and signaling from the structures of two Frizzled cysteine rich domains // *Nature*, 2001, V. 412, P. 86-90.
 2. Voronkov A.E., Baskin I.I., Palyulin V.A., Zefirov N.S., Molecular model of the Wnt protein binding site on the surface of dimeric CRD domain of the hFzd8 receptor. // *Dokl. Biochem. Biophys.*, 2008, Volume 419, Pages 75-78.
 3. SYBYL 7.1, Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.
-

2.10 Calculations

The relative luciferase activity in %, A(%), was calculated by:

$$A (\%) = \frac{X - \overline{\text{neg.contr.}}}{\overline{\text{pos.contr.}} - \overline{\text{neg.contr.}}} \times 100\%$$

where X is the measured luciferase activity of the sample, $\overline{\text{neg.contr.}}$ is the average of the negative controls, and $\overline{\text{pos.contr.}}$ is the average of the positive controls.

The relative luciferase activity in % without activation of the canonical Wnt signaling pathway, B(%), was calculated by:

$$B (\%) = \frac{X}{\overline{\text{neg.contr.}}} \times 100\%$$

where X is the measured luciferase activity of the sample, $\overline{\text{neg.contr.}}$ is the average of the negative controls.

The normalized relative luciferase activity in %, C(%), was calculated by:

$$C (\%) = \frac{X - \overline{\text{neg.contr.}}}{\overline{\text{pos.contr.}} - \overline{\text{neg.contr.}}} \times 100\%$$

$$X = \left(\frac{\text{LUC}}{\text{REN}} \right)_x, \text{ neg.contr.} = \left(\frac{\text{LUC}}{\text{REN}} \right)_{\text{neg.contr.}}, \text{ pos.contr.} = \left(\frac{\text{LUC}}{\text{REN}} \right)_{\text{pos.contr.}}$$

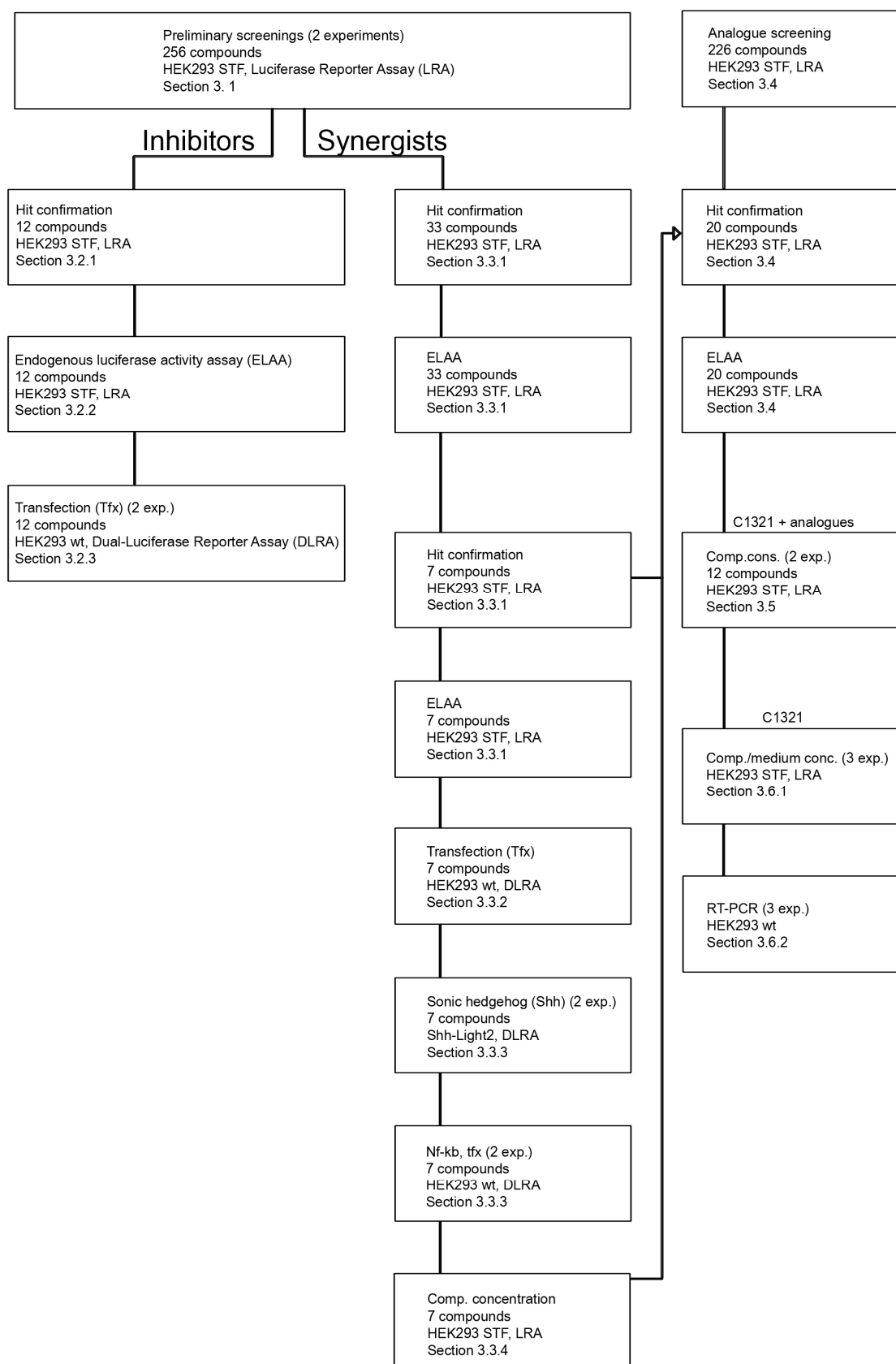
where X is the ratio between the measured luciferase activity of the sample and the measured *Renilla* activity of the sample,
 $\overline{\text{neg.contr.}}$ is the average of the ratios between the measured luciferase activity for the negative controls and the ratios between the measured *Renilla* activity for the negative controls, and
 $\overline{\text{pos.contr.}}$ is the average of the ratios between the measured luciferase activity for the positive controls and the ratios between the measured *Renilla* activity for the positive controls

The strength of the conditioned medium, D, was calculated by:

$$D = \frac{\overline{\text{pos.contr.}}}{\overline{\text{neg.contr.}}}$$

where $\overline{\text{neg.contr.}}$ is the average of the negative controls and $\overline{\text{pos.contr.}}$ is the average of the positive controls.

2.11 Flowchart of methodology



3 Results and Discussion

The aim of the present study was to find small molecules that increased or decreased the binding between Frizzled and Wnt in the canonical Wnt signaling pathway in order to increase or decrease the transcription of target genes.

Prior to the present study, Andrew Voronkov has used an *in silico* based screening (see section 2.9) to find compounds that could possibly increase or decrease the binding between Frizzled and Wnt. From this modeling 256 compounds was chosen for further screening and examination in the laboratory. The structures of these 256 compounds were not revealed prior to the screening and examination, and only a few of the structures were revealed at the end of the examination of these 256 compounds. But due to secrecy until publication at Section for Cellular and Genetic Therapy (Institute of Microbiology, Rikshospitalet, Norway), the author can not reveal the structures in this master thesis.

3.1 Screening of 256 compounds using HEK293 STF cells and Luciferase Reporter Assay

HEK293 STF cells are HEK293 cells with a STF reporter vector stably integrated into the genome. HEK293 cells are very easy to grow and are often used in cell biology research, so the results obtained may easily be compared to other research. The STF reporter vector will be transcribed when the canonical Wnt signaling pathway is activated with a Wnt ligand. The transcription will generate a luciferase enzyme that catalyzes an oxidation of luciferin, which results in emission of light which is measured with a luminometer. By using cells with a STF reporter vector stably integrated into the genome, no transfection had to be performed, and the transcription of target genes could be measured with luciferase activity instead of using RT-PCR (both transfection and RT-PCR are more time consuming methods). One of the advantages of using the Luciferase Reporter Assay on HEK293 cells is that mammalian cells have little or no endogenous luciferase activity.

The purpose of a screening of the compounds using HEK293 STF cells and Luciferase Reporter Assay was to find compounds that increased or decreased the luciferase signal compared to the controls without added compound. An increased or decreased signal may indicate an activating, synergistic or inhibitory effect on the canonical Wnt signaling pathway.

Two separate small scale screenings of the 256 chosen compounds were carried out on HEK293 STF cells. After ~12 hours the cells were stimulated with Wnt-3A conditioned medium and treated with the compounds to a final concentration of 20 μ M (see section 2.4). A Wnt-3A ligand will activate the canonical Wnt signaling pathway by binding to Frz and LRP, and the compounds under investigation were presumed to increase or decrease the binding between Frz and Wnt-3A. The luciferase activity was measured with the Luciferase Assay System ~24 hours after adding the compounds. The luciferase values were calculated relative (in %) to the average of three negative and the average of three positive controls (see section 2.10 A). Wnt-3A treatment alone was used as a positive control, and medium without Wnt-3A (DMEM/FBS/PS, from now referred to as regular cell medium) were used as the negative control. The negative controls show the endogenous luciferase reporter activity.

A way to characterize the activity and thereby the activating properties of the Wnt-3A conditioned medium is to calculate the ratio between the luciferase signal when the cells are treated with conditioned Wnt-3A medium (positive control) and the luciferase signal when the cells are not treated with conditioned Wnt-3A medium (negative control). In these two preliminary screenings the positive to negative ratio varied between 3 and 30. For compounds C1089 to C1202 in the first screening the positive to negative ratio was 3, while the positive to negative ratio was between 16 and 30 for the rest of the first screening and all of the second screening.

Figure 14 shows the average of the two screenings for the 256 compounds, with error bars showing the spread. The luciferase values were calculated relative (in %) to negative and positive controls (see section 2.10 A). Compounds with an average of less than 80 % (inhibitors) or more than 300 % (activators) in the screenings were chosen for hit confirmation. An average relative activation of 80 % is not low enough to be considered as a potent inhibitor, but due to the small amount of potentially inhibitors the limit in the preliminary screenings was set this high. In Figure 14 the 12 possible inhibitors are marked with red while the 33 possible activators are marked with blue. Figure 15 displays the average of the two screenings for these 45 compounds and the error bars showing the spread.

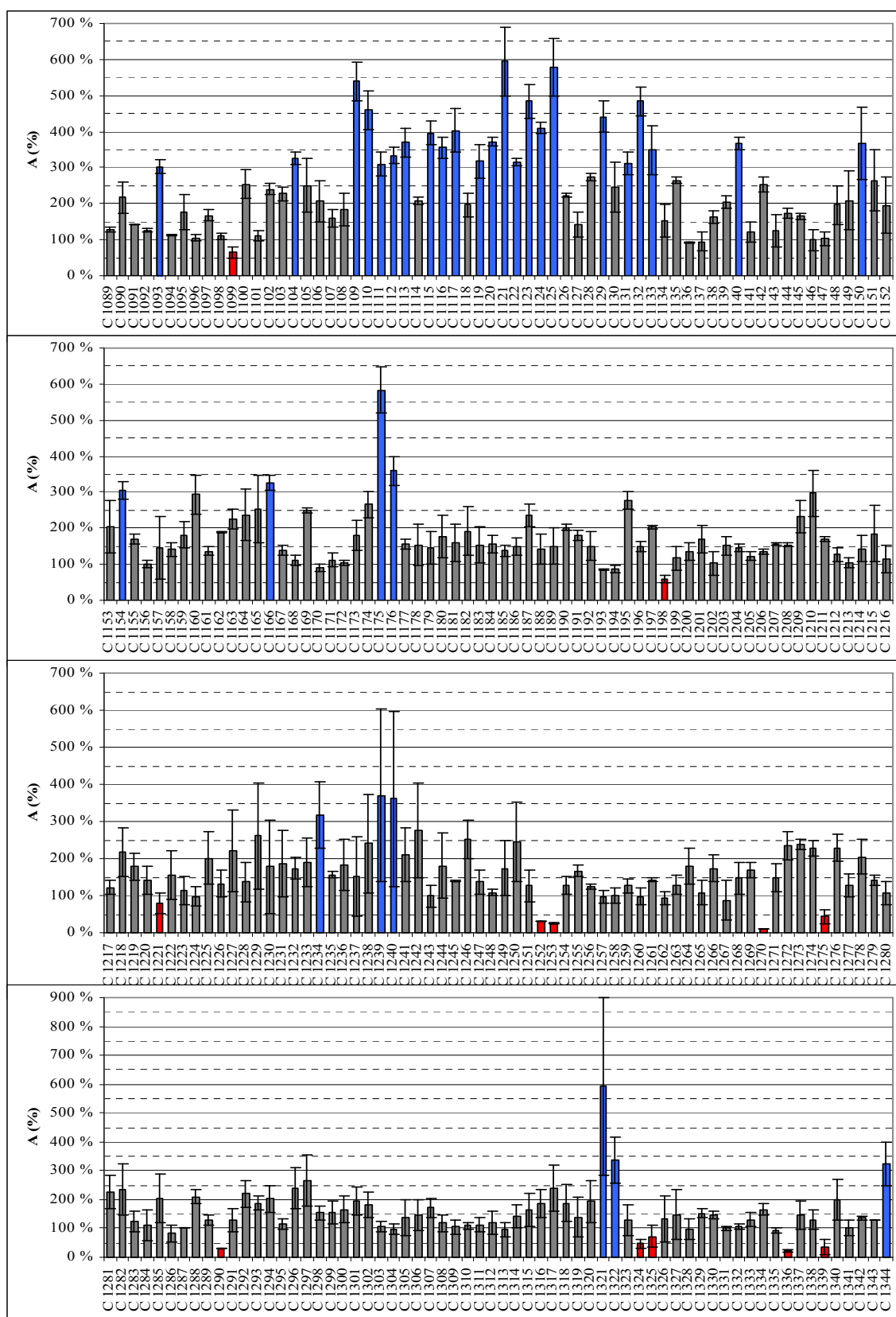


Figure 14 The relative luciferase activity in % (2.10 A) for 256 compounds (two experiments with n=1). The two screenings were performed on HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the average of the two screenings, error bars show the spread. Colored bars indicate the compounds chosen for hit confirmation (blue for activating compounds and red for inhibiting compounds).

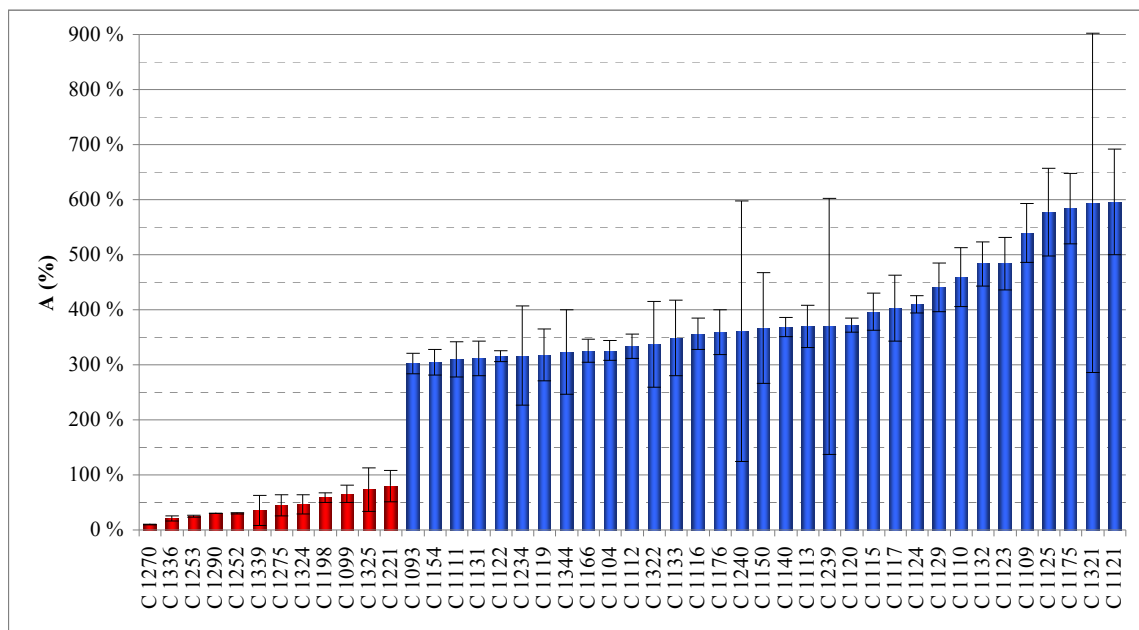


Figure 15 Summary of potential inhibitors and synergists of the canonical Wnt signaling pathway from the screenings, Figure 14. The relative luciferase activity in % (2.10, A) for 45 compounds (two experiments with n=1). The two screenings were performed on HEK293 STF cells, activated with Wnt-3A conditioned medium and added compounds to a final concentration of 20 μ M. Bars show the average of the two screenings, error bars show the spread (blue bars for activating compounds and red bars for inhibiting compounds).

3.2 Inhibitors

The twelve compounds that indicated an inhibitory effect on the canonical Wnt signaling pathway from the two screenings are shown in Figure 16. The luciferase activities in the two screenings were calculated relative to the controls in % (2.10 A), and the compounds with an average below 80 % were chosen for hit confirmation.

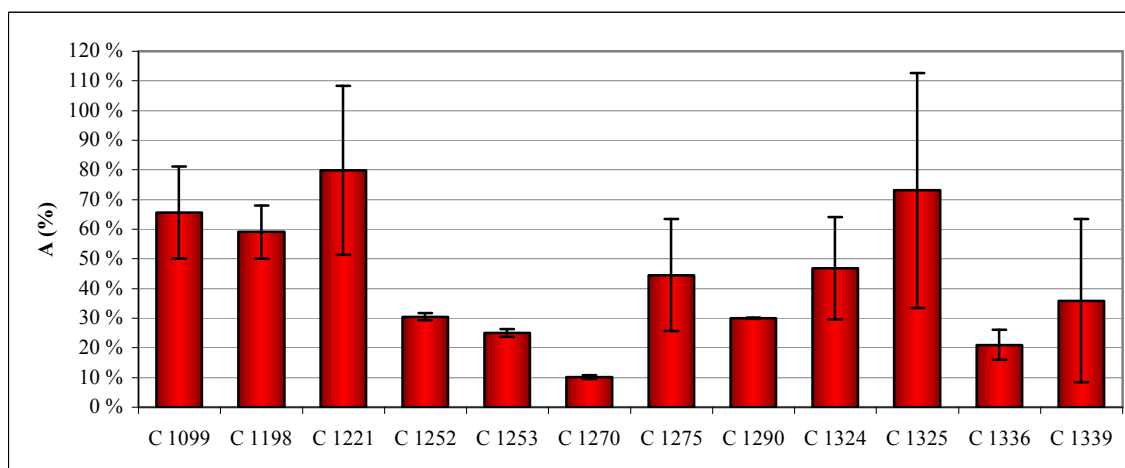


Figure 16 Summary of potential inhibitors of the canonical Wnt signaling pathway from the screenings, Figure 14. The average of the relative luciferase activity in % (2.10 A) for twelve compounds (two experiments with $n=1$). Bars show the average of the two screenings, error bars show the spread.

3.2.1 Hit confirmation of twelve possibly inhibitory compounds

To confirm the results from the two screenings (indicated inhibitory effect on the canonical Wnt signaling pathway), the twelve compounds were examined again with the same cells and method, but in duplicates instead of singlets. For some of the compounds the two screenings also showed high variation % relative activity, and this had to be examined to confirm or dismiss these compounds as inhibitors.

The hit confirmation of the twelve compounds was carried out on HEK293 STF cells. The Wnt signaling pathway was activated with Wnt-3A conditioned medium, and the compounds were added to a final concentration of 20 μ M. Two negative controls with regular cell medium and two positive controls with Wnt-3A conditioned medium were used. The luciferase activities were measured with the Luciferase Assay System, and the activities were calculated relative to positive and negative controls (2.10 A) The positive to negative ratio (2.10 D) was ~50 in this experiment. Figure 17 shows the hit confirmation and the two screenings.

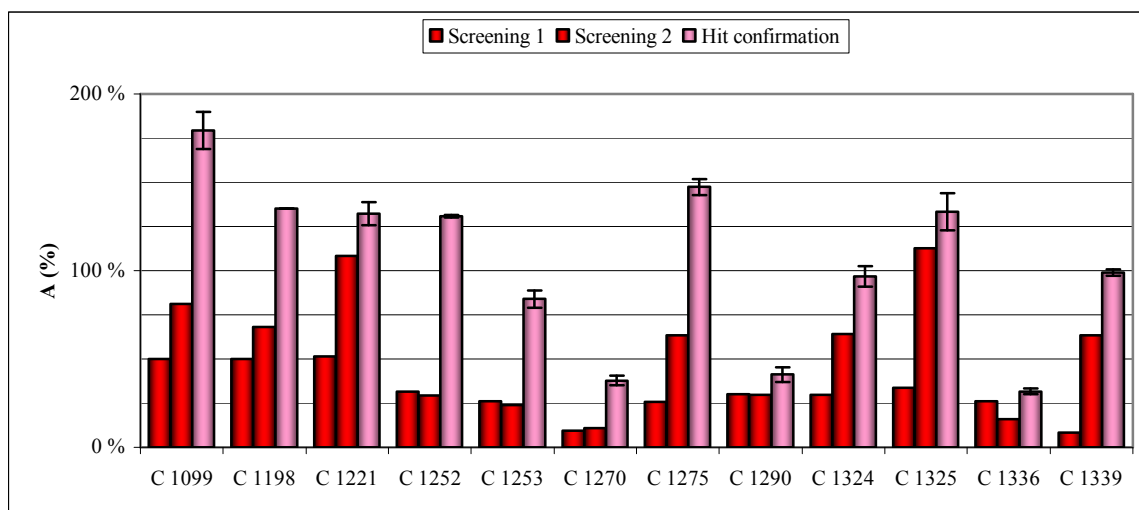


Figure 17 The relative luciferase activity in % (2.10 A) for twelve compounds (two screenings with n=1 and one hit confirmation with n=2). Performed on HEK293 STF cells, activated with Wnt-3A conditioned medium and added compounds to a final concentration of 20 μ M. Red bars show the two screenings. Pink bars show the average of the hit confirmation with error bars showing the spread.

All the compounds gave a higher relative activation in the hit confirmation, and some of the compounds actually showed a synergistic effect. Compound C1270, C1290 and C1336 gave a relative luciferase activity below 50 % for both screenings and the hit confirmation. These three compounds were therefore the most interesting. Because of the varying results for the other compounds, they were less interesting, but all compounds were examined further.

3.2.2 The effect of inhibiting compounds on un-stimulated HEK293 STF cells

In addition to increase or decrease the binding between Wnt and Frz, a compound could affect the canonical Wnt signaling pathway at another level e.g. in the cytoplasm or in the nucleus. It could also affect the luciferase reporter gene by other signaling pathways. The compound could affect the oxidation reaction which provides the light, or the compound could be toxic and decrease cell growth or kill cells.

To explore the effect of the compounds on endogenous luciferase reporter activity HEK293 STF cells with regular cell medium and the compounds at a final concentration of 20 μ M were used. Luciferase Assay System was used to measure the luciferase activity approximately 24 hours after the compounds were added. The luciferase activities were calculated relative to an average of four controls without added compound (2.10 B).

Figure 18 shows the found luciferase activities relative to the controls (in %).

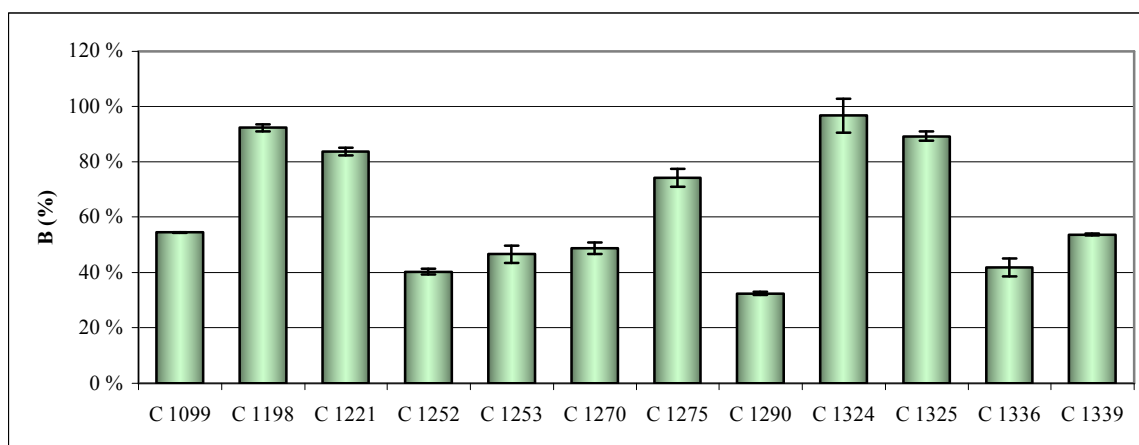


Figure 18 The luciferase activities in % relative to negative controls (2.10 B) for twelve compounds (n=2). The effect on endogenous luciferase activity was explored on HEK293 STF cells with regular cell medium and compounds to a final concentration of 20 μ M. Bars show the average, error bars show the spread.

None of the compounds gave increased luciferase signal compared to the controls. Four of the compounds gave a luciferase signal between 80 % and 100 % of the controls, and the rest of the compounds gave luciferase signals between 30 % and 80 % of the controls. A decreased luciferase signal could be due to non-specific binding (binding at other places than between Frz and Wnt), or due to a toxic effect because of unspecific binding to other

generally important components in a cell. In this master thesis 20 % deviation from the control was regarded as normal variation in the endogenous luciferase activity assay.

The compounds C1270, C1290 and C1336 were the only compounds that gave decreased luciferase signals in both screenings and the hit confirmation. All these compounds also gave a decreased luciferase signal without Wnt, indicating a possibly non-specific or toxic effect.

3.2.3 Transfection with luciferase and *Renilla* plasmids

When using HEK293 STF cells and the Luciferase Assay System, no internal control to normalize the luciferase activity against is used. A decreased or increased luciferase signal could therefore be due to e.g. decreased or increased cell growth. When using the Dual-Luciferase Reporter Assay, a *Renilla* vector may be used as an internal control. A constitutive expression vector like *Renilla* luciferase will be transcribed continuously and lead to emission of light independent of other factors. Unfortunately the author did not have a cell line with both *Renilla* luciferase and firefly luciferase with canonical Wnt signaling reporter vector stably transfected at the time the experiments were performed. Therefore transient transfection with a STF plasmid and a *Renilla* plasmid had to be performed.

This method implies more steps and unfortunately the possibility of more pipetting errors in addition to being a more time consuming method compared to the Luciferase Assay System on the stably transfected cell line HEK293 STF. The HEK293 STF cell line is also clonally selected for high sensitivity (strong signal) for the canonical Wnt signaling pathway, and will therefore give a higher response to activation of the canonical Wnt signaling pathway compared to the HEK293 wt cell line. In addition to this, transfection also cause more stress to the cells.

Despite of these drawbacks, the Dual-Luciferase Reporter Assay will give complementary information about how the compounds affect the canonical Wnt signaling pathway.

Two experiments were performed, each in duplicates. The transient transfection with STF plasmid and *Renilla* plasmid (see section 1.7.3 and 2.6) were performed on HEK293 wt cells. 18 hours after transfection the canonical Wnt signaling pathway was activated by treating the cells with Wnt-3A conditioned medium and the compounds were added to a final concentration of 20 μ M. Two positive controls with Wnt-3A conditioned medium and two negative controls with regular cell medium were used for the first experiment, and four positive and four negative controls were used for the second experiment. The luciferase and *Renilla* activities were measured with the Dual-Luciferase Reporter Assay System.

The ratio between the luciferase activity and the *Renilla* activity for the compounds were calculated relative (in %) to the ratio between the luciferase activity and the *Renilla* activity for negative and positive controls (2.10 C). Figure 19 shows the relative ratios between the luciferase activities and the *Renilla* activities for the twelve compounds.

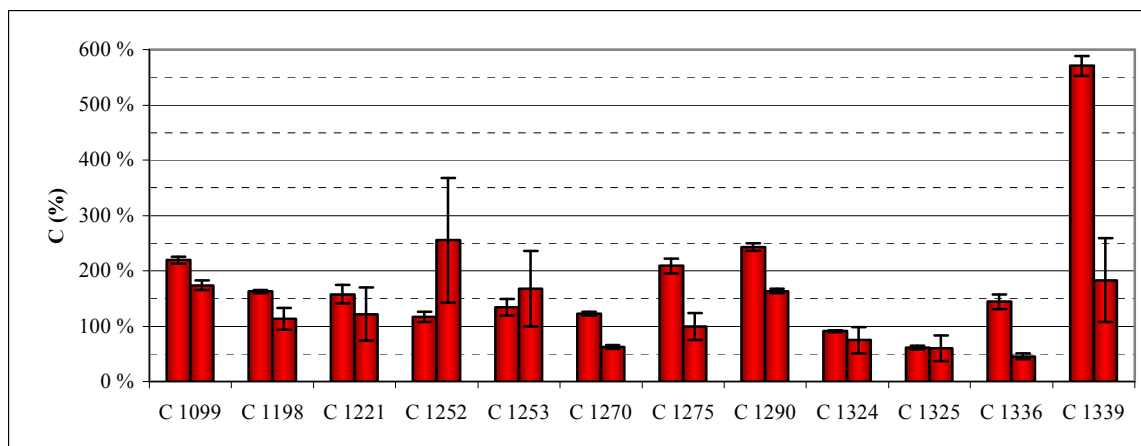


Figure 19 The ratio between the luciferase activity and the *Renilla* activity relative to controls in % (2.10 C) for twelve compounds (two experiments with n=2). HEK293 wt cells transiently transfected with STF plasmids and *Renilla* plasmids, activated with Wnt-3A conditioned medium and added compounds to a final concentration of 20 μ M. Bars show the average, error bars show the spread.

By cell death or decreased cell growth, both the luciferase signal and the *Renilla* signal will be affected. A luciferase to *Renilla* ratio over 100 % relative to controls suggests an activating effect. Contrary to the result from the screenings (see section 3.1), but in accordance with some of the results from the hit confirmation (see section 3.2.1) several of the compounds showed increased activation in the luciferase signal compared to the *Renilla* signal. A luciferase to *Renilla* ratio below 100 % could occur because of inhibited luciferase signaling compared to *Renilla* signaling. Only compound C1336 gave an average relative luciferase to *Renilla* ratio below 50 %, and only in the second experiment.

3.2.4 Concluding remarks

The average of two preliminary screenings indicated an inhibitory effect for twelve of the 256 compounds (average below 80% relative to controls). The hit confirmation gave a higher activation than the screenings for all the compounds, and only three compounds (C1270, C1290 and C1336) gave a relative activation below 80% (25-50%).

This variation could be due to age of the cells, where the cells are in their cell cycle, age and strength of the Wnt-3A conditioned medium, and the stability of the compounds. Crystal formation of the compounds could also be a problem; several of the compounds were very difficult to get fully dissolved.

The endogenous luciferase activity assay showed activity compared to controls below 80% for eight of the compounds. The low activity could be due to non-specific binding or a toxic effect on the cells. Compound C1198, C1221, C1324 and C1325 had activation relative to controls between 80 and 110%, indicating no substantial effect on the endogenous luciferase activity (in this master thesis the author has defined 20 % deviation from the control as normal variation in the endogenous luciferase activity assay).

The transient transfection of *Renilla* and STF plasmids gave activation for several of the compounds, only compound C1324 and C1325 gave decreased relative luciferase to *Renilla* ratios.

The inhibition in the screenings, hit confirmation or transfection did not seem sufficiently consistent to continue with further studies. The affinity and potency of the inhibitors could be improved by finding analogues; this was not done in this master thesis.

3.3 Activators

The 33 compounds that indicated a synergistic effect on the canonical Wnt signaling pathway from the two screenings (see section 3.1) are summarized in Figure 20. The luciferase activities were calculated in % relative to positive and negative controls (see section 2.10 A), and the compounds with an average above 300 % were chosen for further examination.

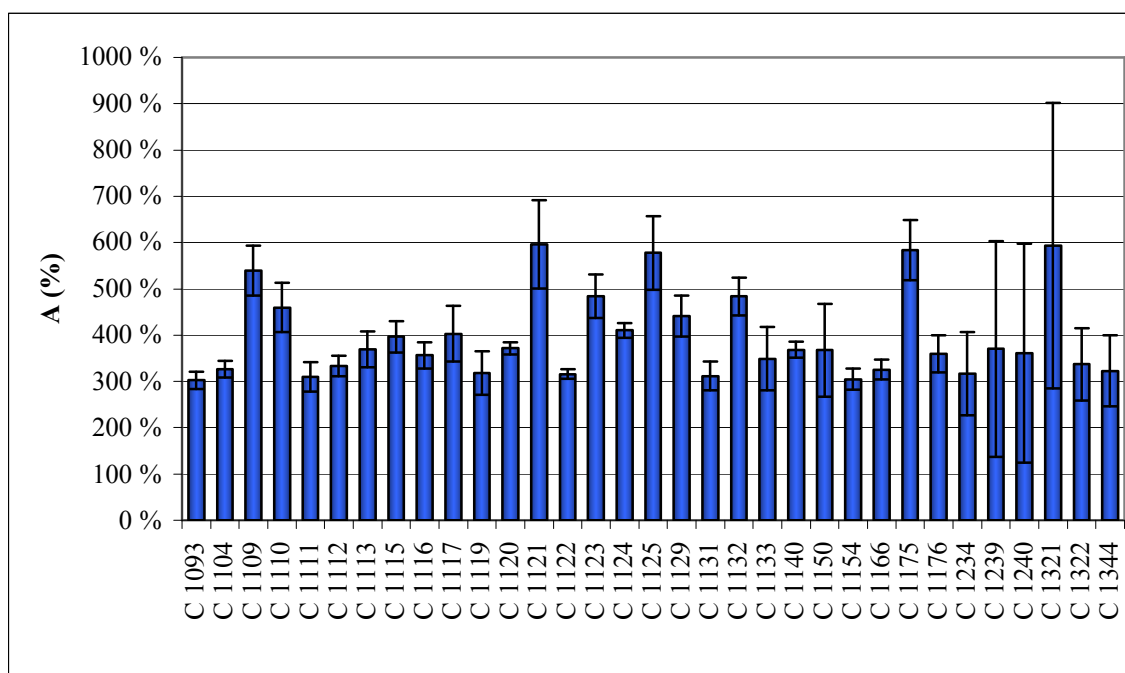


Figure 20 Summary of potential synergists of the canonical Wnt signaling pathway from the screenings, Figure 14. The average of the relative luciferase activity in % (2.10 A) for the 33 compounds (two experiments, n=1). Bars show the average of the two screenings, error bars show the spread.

3.3.1 Hit confirmations and endogenous luciferase activity assays

To confirm the results from the two screenings, the 33 compounds were examined again with the same cells and method, but now in duplicates.

To explore the effect of the compounds on the endogenous luciferase activity of the cells (i.e. the luciferase activity in the cells without activating the canonical Wnt signaling pathway with Wnt-3A conditioned medium), would give information about the specificity of the compounds. As mentioned in section 3.2.2 a compound could affect the canonical Wnt signaling pathway at other places than in the binding between Wnt and Frz, and it could also affect the luciferase reporter gene by other signaling pathways. The compound could influence the oxidation reaction which provides the light, or the compound could decrease cell growth or kill cells by binding to and disturb important cellular functions. The compounds could also possibly be activators of the canonical Wnt signaling pathway without Wnt present. Only synergists and inhibitors of the canonical Wnt signaling pathway that increased or decreased the binding between Wnt ligand and Frz receptor were sought in this study. Therefore the 33 compounds were also examined for their effect on the cells without Wnt ligands present.

The hit confirmation experiment and the study of possible effect of the compounds on endogenous luciferase activity were carried out in duplicates on HEK293 STF cells. For the hit confirmation experiment the Wnt signaling pathway was activated with Wnt-3A conditioned medium, and to examine the effect of the compounds on endogenous luciferase activity only regular cell medium were used. In both experiments the compounds were added to a final concentration of 20 μ M.

Seven positive controls with Wnt-3A conditioned medium and seven negative controls with regular cell medium were used for the hit confirmation experiment, and the positive to negative ratio was 97. Eight controls with regular cell medium were used to investigate the effect on endogenous luciferase activity.

The luciferase activities were measured with the Luciferase Assay System. Figure 21 shows the luciferase activities calculated in % relative to the controls (2.10 A and B).

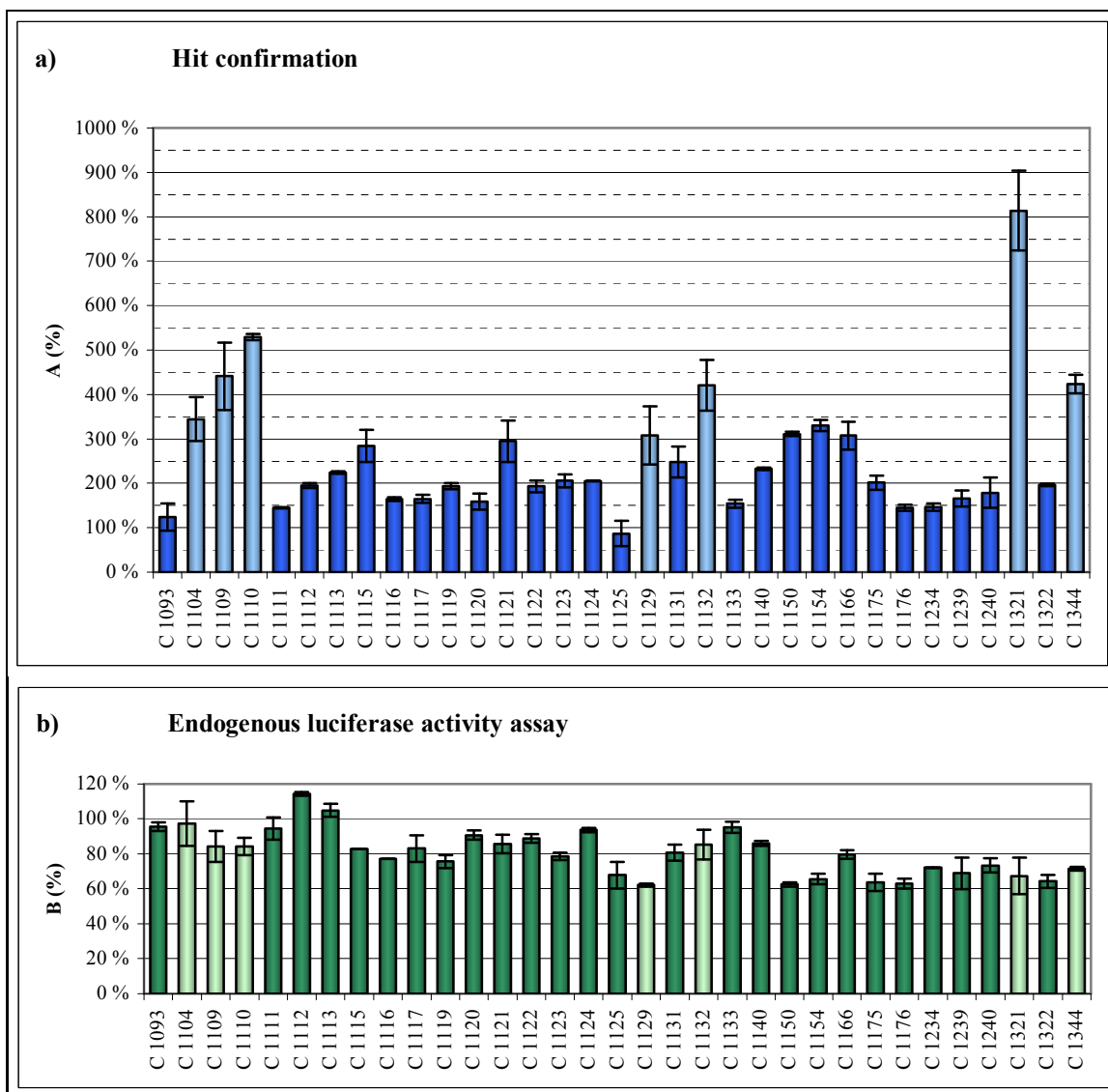


Figure 21 Hit confirmation experiment and endogenous luciferase activity assay.

a) The relative luciferase activity in % (2.10 A) for 33 compounds. Performed on HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread (n=2). Light blue colored bars indicate the compounds chosen for further examination.

b) The relative luciferase activity in % (2.10 B) for 33 compounds. Performed on HEK293 STF cells with regular cell medium and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread (n=2). Light green colored bars indicate the compounds chosen for further examination.

Many of the compounds gave relative luciferase activities below 300 % in the hit confirmation experiment, and only the compounds with at least one replicate value above 350 % were chosen for further investigation (seven compounds, colored light blue in Figure 21a).

Compound C1321 gave the best relative activation of the compounds, with an average relative luciferase activity above 800 %. This compound had a relative luciferase activity of ~300 % and ~900 % in the two separate screenings.

From the investigation of the effect of the compounds on endogenous luciferase activity, two of the compounds had an average relative luciferase activity between 100 and 120 % and 16 of the compounds had an average between 80 and 100 %. This could be due to normal variation in the cells (in this master thesis the author has defined 20 % deviation from the control as normal variation in the endogenous luciferase activity assay). 15 of the compounds had an average relative luciferase activity between 60 and 80 %, and this could indicate non-specific or toxic effects in the cells.

Of the seven chosen compounds from the hit confirmation experiment, three of them (C1129, C1321 and C1344) had an average relative luciferase activity between 60 and 80 %.

The seven chosen compounds with at least one replicate value above 350 % from the hit confirmation experiment were re examined in duplicates in a second hit confirmation experiment and endogenous luciferase activity assay. A summary of the two screenings, the two hit confirmation experiments and the two endogenous luciferase activity assays for the seven compounds are shown in Figure 22.

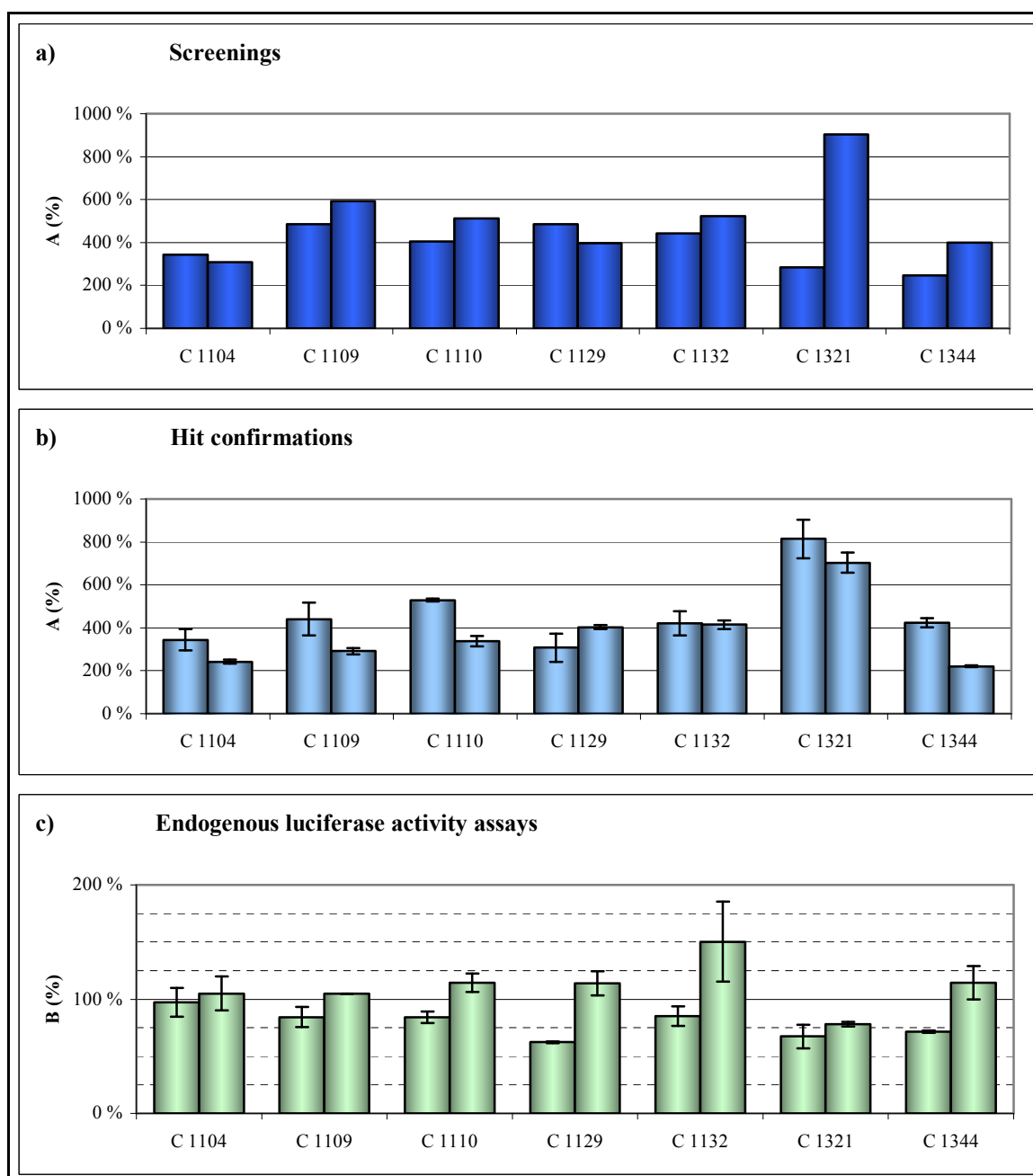


Figure 22 Summary of the screenings, hit confirmation experiments and the endogenous luciferase activity assays for the seven compounds that indicate a synergistic effect on HEK293 STF cells.

a) The relative luciferase activity in % (2.10 A) of the two screenings performed on HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the two screenings (two experiments with $n=1$). See section 3.1.

b) The relative luciferase activity in % (2.10 A) of the two hit confirmation experiments performed on HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread ($n=2$). See section 3.3.1.

c) The relative luciferase activity in % (2.10 B) in HEK293 STF cells with regular cell medium and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread ($n=2$). See section 3.3.1.

The compounds shown in Figure 22 gave a quite similar activation in both the screening and the hit confirmation experiments. As mentioned earlier some variations are normal and due to differences in the age of the cells, where the cells are in their cycle, the age and strength of the Wnt-3A conditioned medium, and the stability of the compounds. But despite some variations, the screening and hit confirmation experiments for these compounds gave quite similar responses.

Most of the compounds do not show substantial decrease in the luciferase signal when added to cells with regular cell medium, and this indicates little or no non-specific luciferase activity or toxic effect on the cells. Even though compound C1321 had a decreased luciferase signal of approximately 25 % in the endogenous luciferase activity assay, the activation found in the screening and hit confirmation experiments were so high that this compound was still very interesting. The specificity and toxicity of this compound should be examined further.

3.3.2 Transfection with luciferase and *Renilla* plasmids

As mentioned in section 3.2.3, the Luciferase Assay System used on HEK293 STF cells does not provide an internal control for normalization of the luciferase activity (but has other benefits, see section 3.2.3). The Dual-Luciferase Reporter Assay System, utilizing *Renilla* luciferase and firefly luciferase contain such an internal control by measuring both sequentially from a single sample (see section 1.7.4 and 2.7), and normalizing firefly luciferase activity against *Renilla* luciferase activity (which is a measure of the transfection rate and the viability of the cells).

After transient transfection of Super TOP-Flash plasmids and *Renilla* plasmids (see section 2.6) into HEK293 wt cells, the seven compounds were added to a final concentration of 20 μ M and the canonical Wnt signaling pathway were activated with Wnt-3A conditioned medium. Three positive controls with Wnt-3A conditioned medium and four negative controls with regular cell medium were used. The luciferase and *Renilla* signals were measured with the Dual-Luciferase Reporter Assay System (see section 2.7). The ratio between the luciferase signal and the *Renilla* signal for the compounds were normalized relative to the ratio between the luciferase signal and the *Renilla* signal for negative and positive controls (2.10 C), and the results are shown in Figure 23.

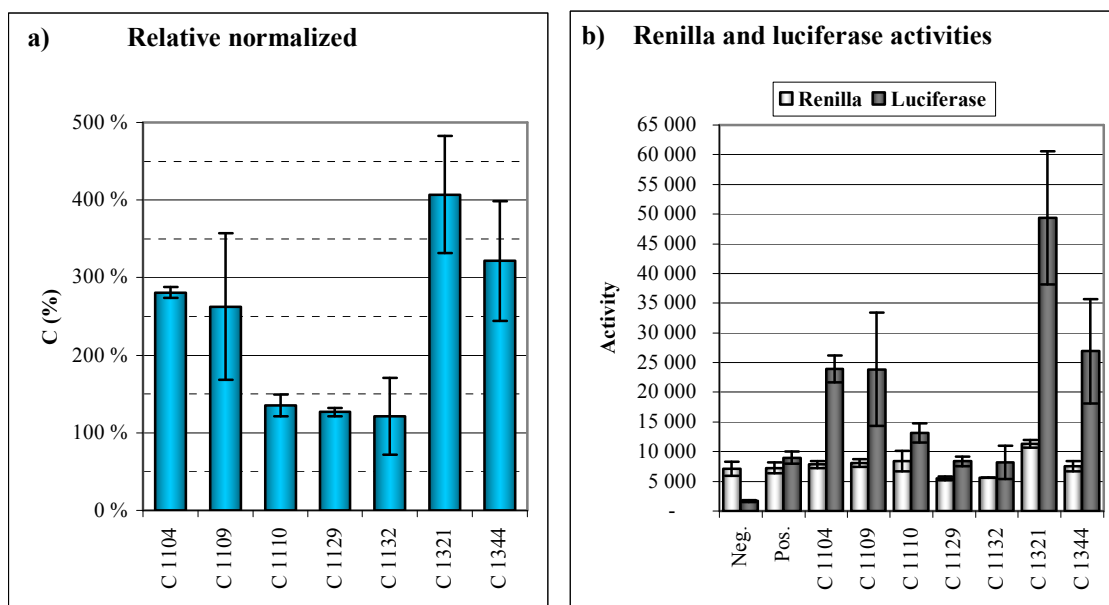


Figure 23

a) The ratio between the luciferase activity and the *Renilla* activity relative to controls in % (2.10 C) for seven compounds (n=2) in HEK293 wt cells transiently transfected with STF plasmids and *Renilla* plasmids, activated with Wnt-3A conditioned medium and added compounds to a final concentration of 20 μ M. Bars show the average, error bars show the spread.

b) The luciferase activity and the *Renilla* activity for seven compounds (n=2) in HEK293 wt cells transiently transfected with STF plasmids and *Renilla* plasmids, activated with Wnt-3A conditioned medium and added compounds to a final concentration of 20 μ M. Bars show the average, error bars show the spread.

Compounds C1110, C1129 and C1132 did not show substantial decrease or increase in the activity ratio compared to the ratio of the controls. Figure 23 b shows that the luciferase activities actually did not increase substantially compared to positive controls for compound C1110, C1129 and C1132 in this experiment, but the *Renilla* values were normal for compound C1110. C1129 and C1132 had a somewhat lower *Renilla* signal compared to the rest of the compounds. This indicates that the cell vitality and the transfection efficiency were not the problem for C1110, but could have some small effect for compound C1129 and C1132. Crystal formation of the compounds could be one reason for these results and another reason could be the cell line. The cell line HEK293 STF is as mentioned clonally selected for high sensitivity.

Compound C1104, C1109, C1321 and C1344 gave an activation compared to the controls, confirming the results from the screenings and hit confirmation experiments.

3.3.3 Specificity

From the screenings and hit confirmation experiments compounds C1104, C1109, C1110, C1129, C1132, C1321 and C1344 all indicated an activating effect in the canonical Wnt signaling pathway by giving activation in the luciferase signaling relative to the controls. The endogenous luciferase activity assays showed that the compounds did not give an activating effect without Wnt, indicating a possibly synergistic effect. Transfection of luciferase and *Renilla* plasmids into HEK293 wt cells did not give normalized relative activation for compound C1110, C1129 and C1132, but they were still examined further.

To check the specificity of the compounds for the canonical Wnt signaling pathway, they were further examined for their effect on the Shh signaling pathway and the NF- κ B signaling pathway (which is also receptor activated pathways). To be used as pharmacological substance, the small molecule needs to be specific in targeting the canonical Wnt signaling pathway. The Shh signaling pathway is one of the most important developmental signaling pathways in the body, and NF- κ B strongly controls the immune and inflammatory responses in the body. Therefore it is important that these signaling pathways are not influenced by the chosen compound.

In addition to affecting these two signaling pathways, the effect of the compounds on the firefly luciferase was examined. If the compounds affect the oxidation process, e.g. by reacting with the luciferin directly or indirectly in some way, the relative luciferase activity would be increased in the Shh signaling pathway and the NF- κ B signaling pathway as well¹⁰⁹.

The Shh signaling pathway

Two separate experiments were performed, each in duplicates. Shh-Light2 cells containing the integrated variant of the GLI-responsive firefly luciferase reporter gene and the *Renilla* luciferase gene which provide constitutive expression of *Renilla* luciferase were used. The compounds were added to the cells to a final concentration of 20 μ M and the Shh signaling pathway were activated with conditioned Shh-medium (see section 2.5). Four negative controls with regular cell medium and four positive controls with conditioned Shh-medium were used. The luciferase and *Renilla* activity was assayed with Dual-Luciferase Reporter Assay System with Stop&Glo ~48 hours after adding the compounds.

The ratio between the luciferase activity (LUC) and the *Renilla* activity (REN) were calculated relative to the ratio of the controls (see section 2.10 C), and Figure 24 shows the results.

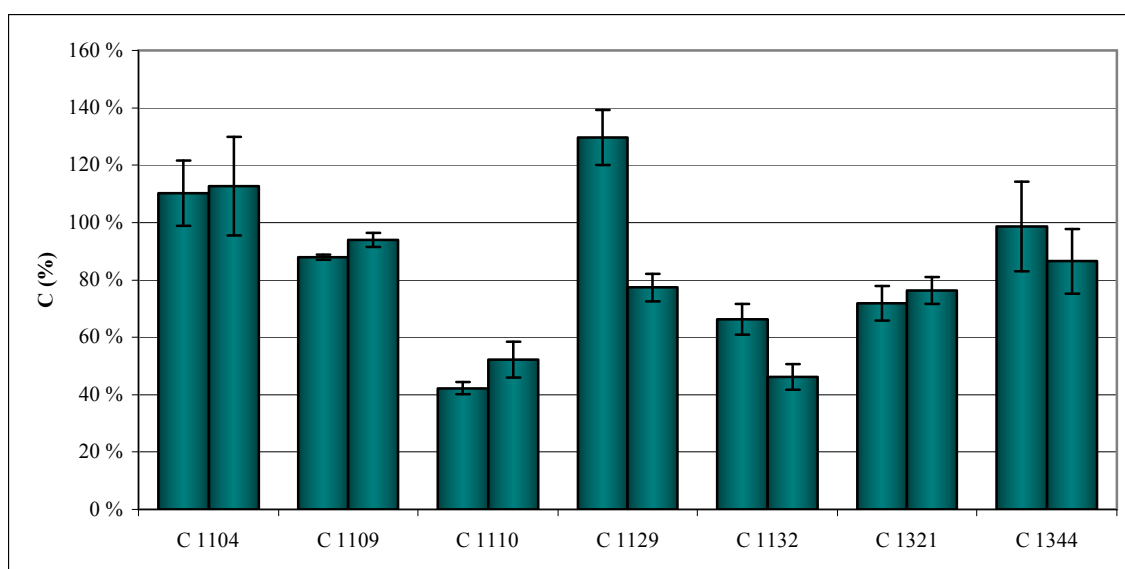


Figure 24 The ratio between the luciferase activity and the *Renilla* activity relative to controls in % (2.10 C) for seven compounds. Shh-Light2 cells were added compounds to a final concentration of 20 μ M and activated with conditioned Shh-medium. Bars show the average, error bars show the spread (two experiments with n=2).

Two compounds (C1110 and C1132) had an average relative ratio between 40 and 70 %. This could indicate some inhibitory effect on the Shh signaling pathway or a toxic effect on the cells. None of compounds showed a substantial normalized relative activation, indicating no considerable activation in any of the pathway components of the Shh signaling pathway.

The NF- κ B signaling pathway

Two separate experiments were performed, each in duplicates. HEK293 wt cells were transiently transfected with NF- κ B-luc plasmids and *Renilla* plasmids (see section 2.6). After 18 hours the compounds were added to a final concentration of 20 μ M and the NF- κ B signaling pathway were activated with TNF- α medium. Four negative controls with regular cell medium and four positive controls with TNF- α medium were used. The luciferase and *Renilla* activity was assayed with Dual-Luciferase Reporter Assay System with Stop&Glo ~24 hours after adding the compounds.

The ratio between the luciferase activity (LUC) and the *Renilla* activity (REN) were calculated relative to the ratio of the controls (see section 2.10 C), and Figure 25 shows the results.

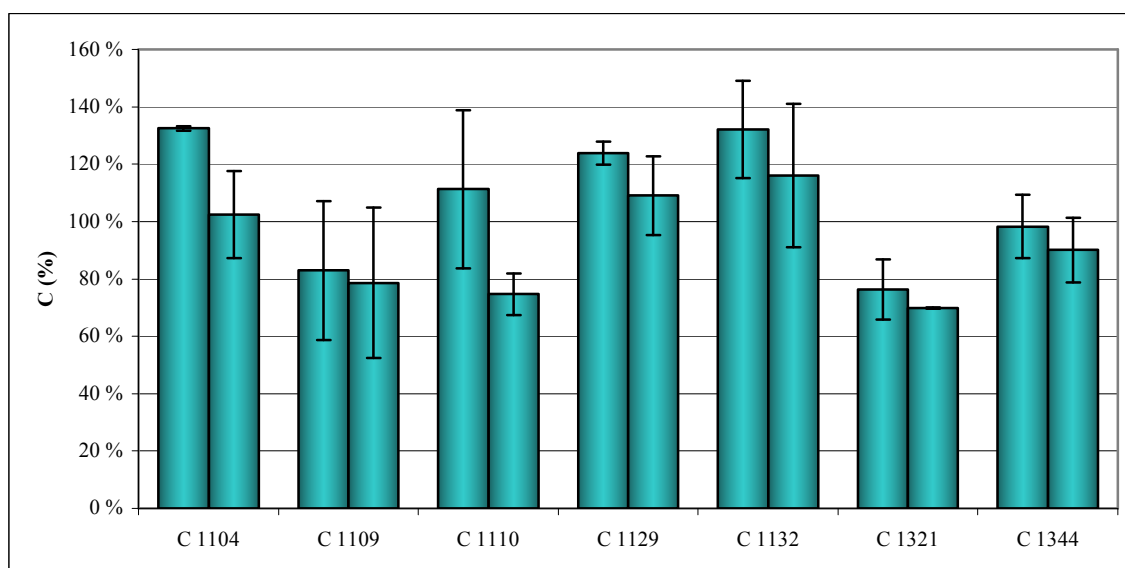


Figure 25 The ratio between the luciferase activity and the *Renilla* activity relative to controls in % (2.10 C) for seven compounds. HEK293 wt cells were added compounds to a final concentration of 20 μ M and the signaling pathway were activated with TNF- α medium. Bars show the average, error bars show the spread (two experiments with n=2).

None of the compounds showed substantial increase or decrease in the ratio compared to controls, which indicates that the compounds did not have any considerable effect on any of the pathway components in the NF- κ B signaling pathway.

3.3.4 Examination of compound concentration

The seven chosen compounds gave increased relative activation compared to controls when tested on HEK293 STF cells, but some of them indicated a non-specific or toxic effect by giving a decreased luciferase to *Renilla* ratio in the transfection (section 3.3.2) and when tested on the Shh signaling pathway (section 3.3.3). High concentration of compounds could be the reason for the possibly toxic effect; therefore different concentrations of the compounds were investigated. If a compound gave high relative activation at low concentrations, this could indicate specific and high affinity for the binding between Wnt and Frizzled.

To explore the effect of different concentrations of a compound on the HEK293 STF cells with Wnt-3A conditioned medium, four different concentrations were used (final concentration of 0.5, 1, 5, 10 and 20 μM). Eight positive controls with Wnt-3A conditioned medium and eight negative controls with regular cell medium were used. The luciferase activities were measured with the Luciferase Assay System. The positive to negative ratio was 154 (see section 2.10 D). Figure 26 shows the results calculated relative to positive and negative controls (see section 2.10 A).

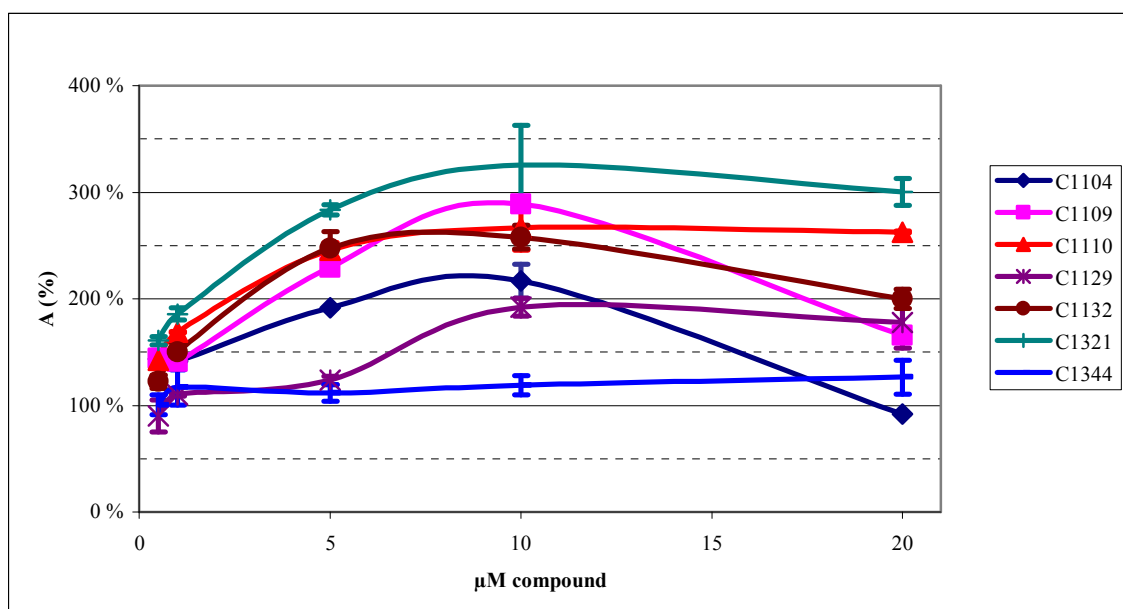


Figure 26 The relative luciferase activity in % (2.10 A) for seven compounds. Performed on HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 0.5, 1, 5, 10 and 20 μM . The trend lines are drawn between average values and error bars show the spread (n=2).

The activation of the luciferase signals was less than in the screening and hit confirmation experiments, but still a small or in average increase in the signal was observed. As a general trend, the signal increased from 0.5 to 10 μ M for all the compounds, but with a decrease in signal for 20 μ M. The lower signal for 20 μ M compared to 10 μ M could be explained by a toxic effect of the compounds on the cells.

Compound C1321 again gave the highest relative activation at all concentrations, reflecting its potency in promoting binding of Wnt-3A to Frz.

3.3.5 Concluding remarks

From the 33 possible activators chosen from the two screening experiments, with an average above 300 % relative to positive and negative controls, seven compounds gave at least one measurement above 350 % in the first hit confirmation experiment and were selected for further examination. The second hit confirmation experiment also gave good increased luciferase activity, and the toxicity of the compounds did not seem to be considerable.

By using transfection of *Renilla* and luciferase plasmids activation in the *Renilla* to luciferase ratio was found for C1104, C1109, C1321 and C1344, but not for C1110, C1129 and C1132.

The specificity of the compounds for the canonical Wnt signaling pathway seemed to be very good. C1110 and C1132 appeared to inhibit the Shh signaling pathway to some extent, but none of the compounds gave activation compared to the controls. The ratio between luciferase and *Renilla* when the compounds were investigated for effect on the NF- κ B signaling pathway did not indicate activation or inhibition.

Increased concentration, up to 10 μ M final concentrations of the compounds, gave an increased activation of luciferase signaling. The decrease in activation for 20 μ M compound can be explained by non-specific or toxic effects on the cells.

3.4 Compound analogues – screening, hit confirmation experiment and endogenous luciferase activity assay

The seven compounds that gave increased activation in the luciferase signal when added to HEK293 STF cells with Wnt-3A conditioned medium were used for further modeling and analogue development by Andrew Voronkov, who suggested 226 analogue compounds.

The analogue development was performed to find compounds that gave even higher activation at lower concentrations (higher specificity and better binding properties) would increase the potency. From the experimental data resulting in finding the seven compounds, the *in silico* modeling was adjusted and analogues which in theory could increase the binding between Wnt and Frizzled even more were found.

The screening of these 226 new compounds was done once in duplicates. HEK293 STF cells were added compounds to a final concentration of 20 μ M, and the canonical Wnt signaling pathway was activated with Wnt-3A conditioned medium. Four negative controls with regular cell medium and four positive controls with Wnt-3A conditioned medium were used. The luciferase activity was measured with the Luciferase Assay System, and the luciferase values were calculated relative to the average of the controls (2.10 A). The positive to negative ratio was 50 (2.10 D).

Figure 27 shows the 226 compounds sorted by average relative luciferase activation.

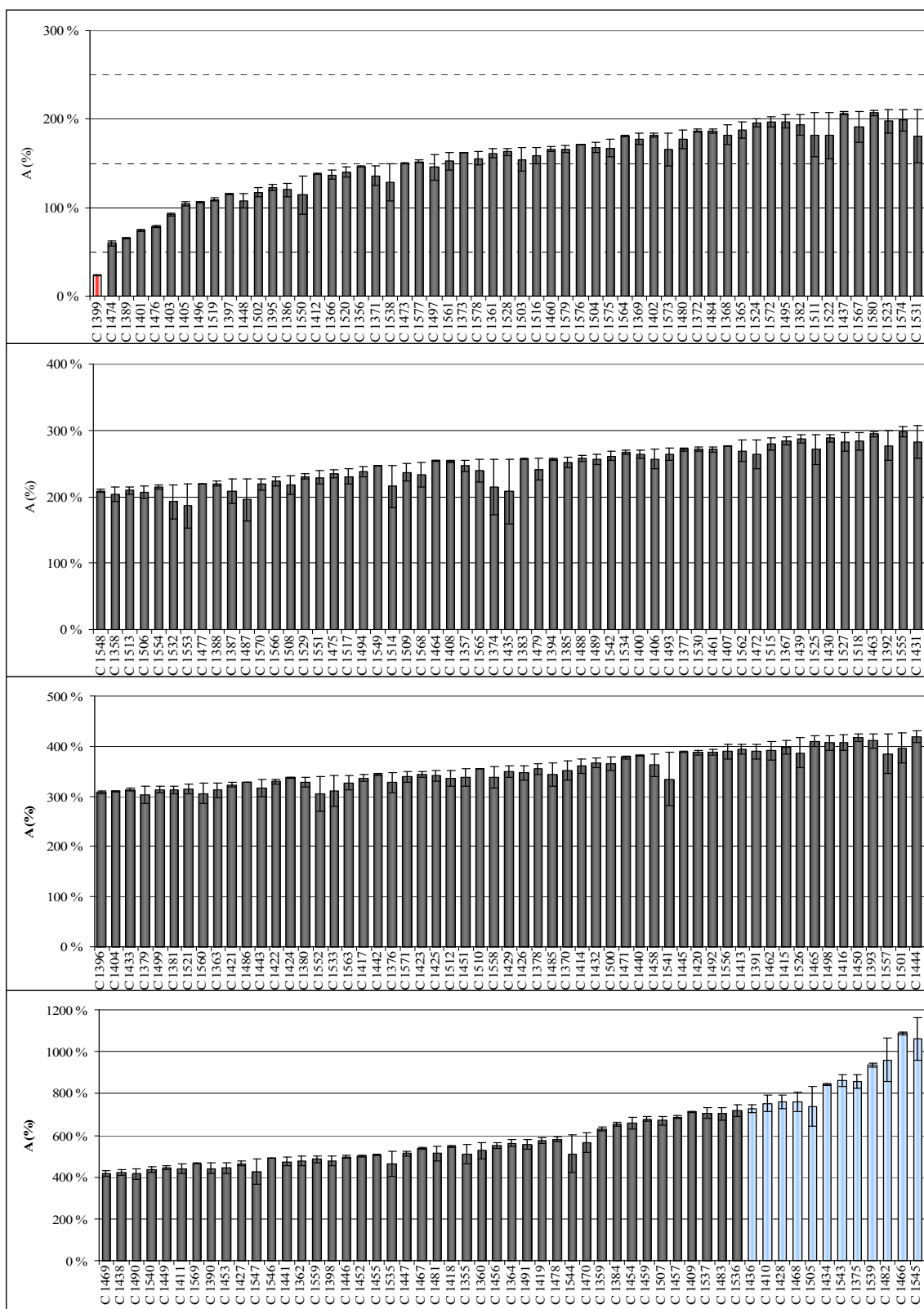


Figure 27 The relative luciferase activity in % (2.10 A) for 226 compounds in HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread (n=2). Light red and light blue colored bars indicate the compounds chosen for further examination.

Some of the compounds gave a decreased relative luciferase signal, and the one with the lowest signal (C1399) was chosen for further examination. The twelve compounds that gave the highest average relative luciferase activation were also chosen for further investigation.

The chemotypes for the seven compounds from the first screening experiments (C1104, C1109, C1110, C1129, C1132, C1321 and C1344) and of the thirteen compounds from the second screening experiment (C1410, C1428, C1434, C1436, C1466, C1468, C1485, C1505, C1543, C1545) was revealed to the author. Compound C1104, C1109 and C1110 belong to the same chemotype, and C1109 and C1110 are quite similar. Compound C1399 belong to the same chemotype as C1321, as do also 11 of the compounds that gave the best activation. C1539 was actually revealed to be the same compound as C1321. Compound C1375 belong to the same chemotype as C1132.

The structures of the seven compounds from the first screening (C1104, C1109, C1110, C1129, C1132, C1321 and C1344) and of the ten compounds similar to C1321/C1539 (C1410, C1428, C1434, C1436, C1466, C1468, C1485, C1505, C1543, C1545) were revealed to the author, but can not be revealed in this thesis.

To confirm the screening results and to compare the new compounds to the former compounds, the twenty compounds were re-examined in duplicates. HEK293 STF cells were added compounds to a final concentration of 20 μ M, and the canonical Wnt signaling pathway was activated with Wnt-3A conditioned medium. Four negative controls with regular cell medium and four positive controls with Wnt-3A conditioned medium were used. The luciferase activities were measured with the Luciferase Assay System. The positive to negative ratio was 35 (2.10 D).

The effects of the 20 compounds on endogenous luciferase activity were also examined in duplicates. HEK293 STF cells were added regular cell medium and compounds to a final concentration of 20 μ M. Four controls with regular cell medium were used, and the Luciferase Assay System was used to measure the activation.

Figure 28 shows the results from the hit confirmation experiment and the endogenous luciferase activity assay, sorted by chemotype of the compounds (light blue and light green bars point out the compounds included from the second modeling experiment).

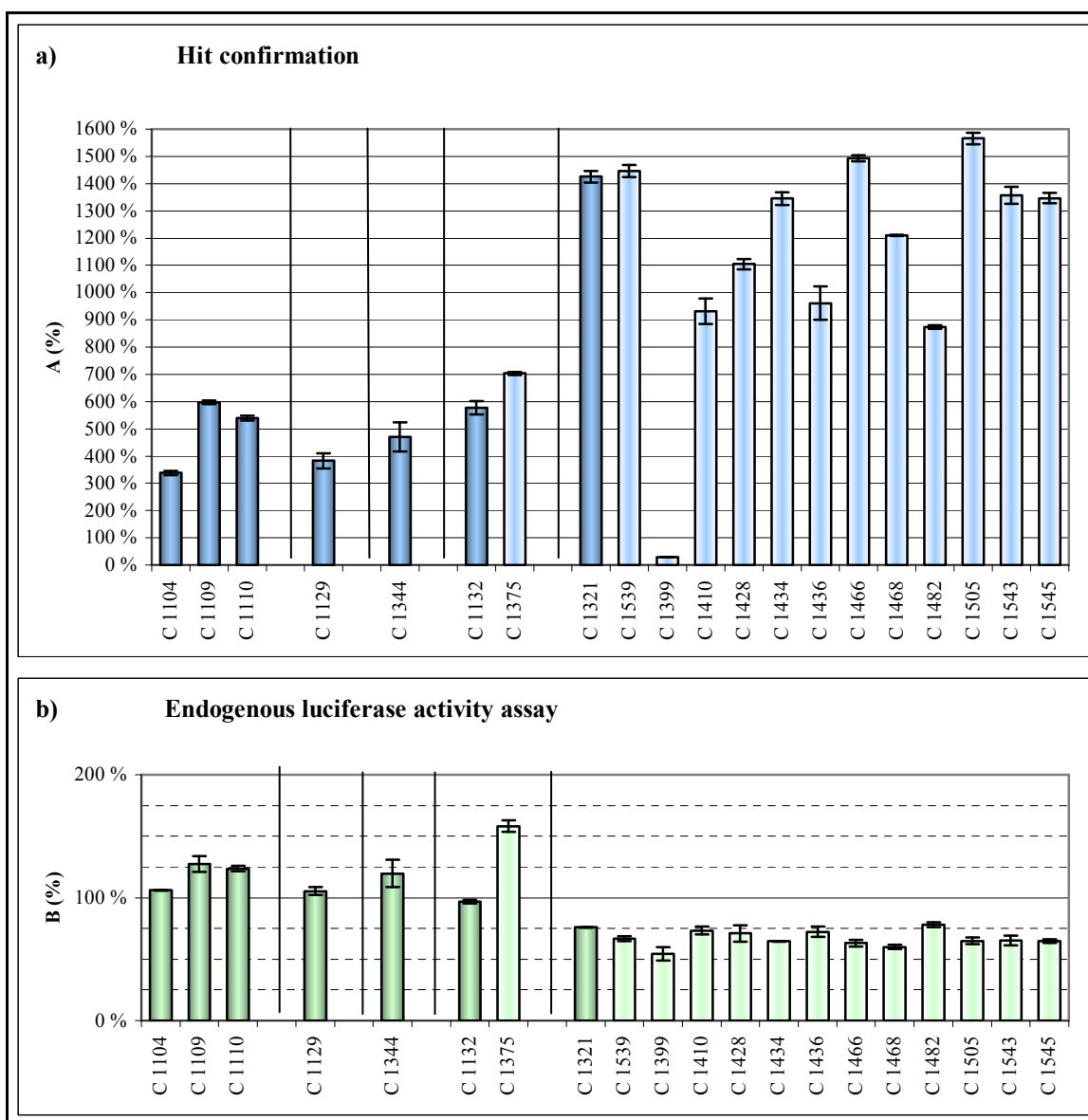


Figure 28 Hit confirmation experiment and endogenous luciferase activity assay of seven compounds from the first screenings and 13 compounds from the last screening, sorted by chemotypes.

a) The relative luciferase activity in % (2.10 A) for 20 compounds in HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread (n=2). Light blue colored bars indicate the compounds included from the second modeling experiment.

b) The relative luciferase activity in % (2.10 B) for 20 compounds in HEK293 STF cells with regular cell medium and added compounds to a final concentration of 20 μ M. Bars show the average and error bars show the spread (n=2). Light green colored bars indicate the compounds included from the second modeling experiment.

A compound that gave an inhibition of the luciferase signal (C1399) showed quite a toxic or non-specific effect on the cells (relative activation ~50 %), and was not chosen for further studies. The best activators were all from the same chemotype as C1321, and even though they gave quite a relative reduction in the luciferase signal without Wnt-3A conditioned medium (endogenous luciferase activity assay) they were still chosen for further examination.

3.5 C1321 and analogues

To investigate the effect of different concentrations of compounds (see section 3.3.4), C1321/C1539 and the ten compounds with the same chemotype were examined twice in duplicates. HEK293 STF cells were added compounds to a final concentration of 1, 5, 10 and 20 μM and the canonical Wnt signaling pathway was activated with Wnt-3A conditioned medium. Four positive controls with Wnt-3A conditioned medium and four negative controls with regular cell medium were used, and the positive to negative ratio were approximately 20 in the first experiment and approximately 35 in the second experiment (2.10 D). The luciferase activities were measured with the Luciferase Assay System, and the values were calculated relative to positive and negative controls. Figure 29 shows the results from this examination of concentration dependency.

For all the compounds, the luciferase signal increased with increasing compound concentration. For some of the compounds the luciferase signal decreased a little from 10 μM to 20 μM , and this could be due to a toxic reaction or non-specific binding because of high concentration.

Compounds C1321 and C1539 is the same compound, but C1539 gave a somewhat higher luciferase signal in the first experiment. In the second experiment the signals were generally lower, and this could among other be due to the stability of the compound. The concentration of the Wnt-3A conditioned medium could also have affected the signal level.

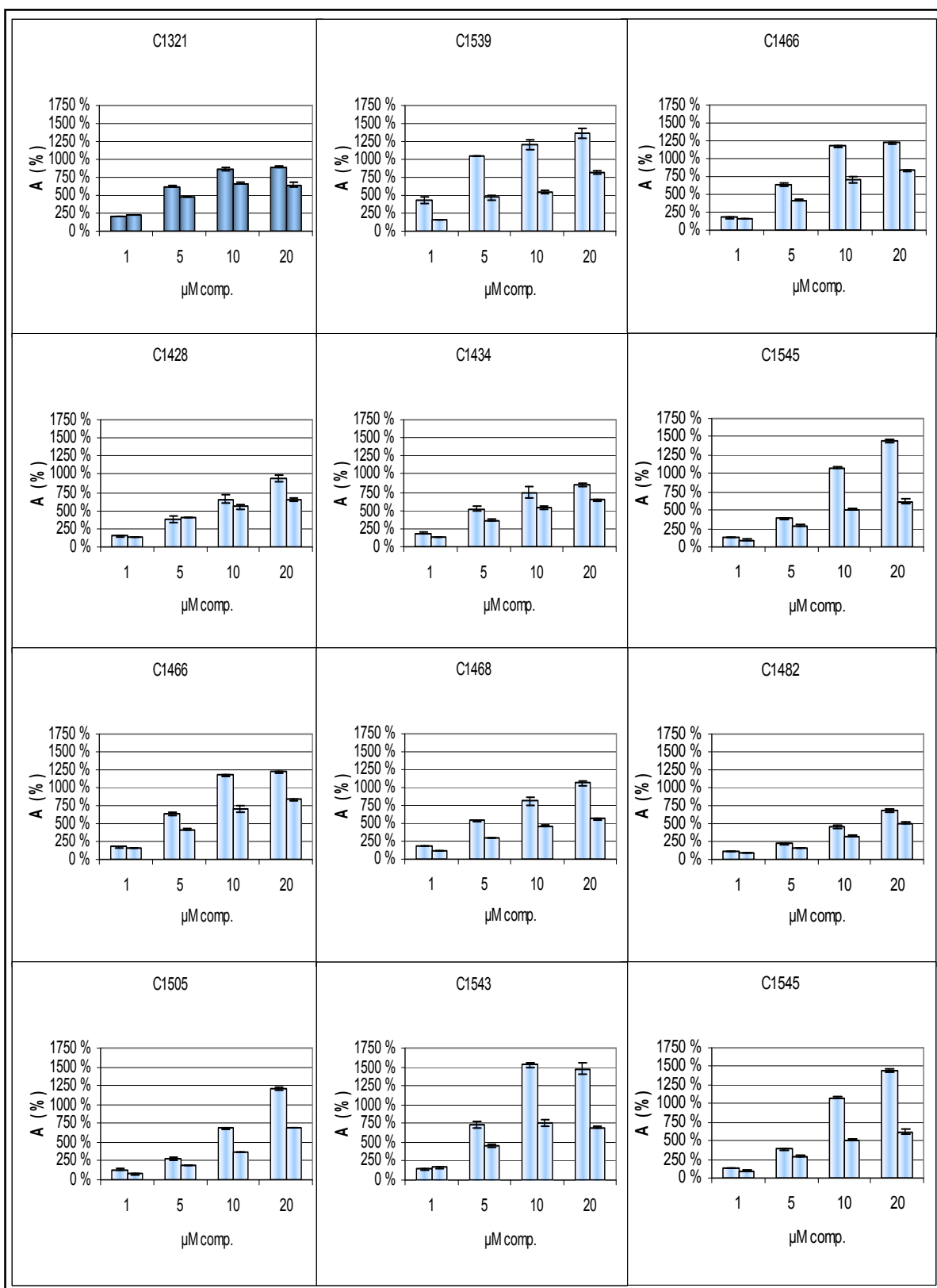


Figure 29 The relative luciferase activity in % (2.10 A) for 12 compounds in HEK293 STF cells, activated with Wnt-3A conditioned medium, and added compounds to a final concentration of 1, 5, 10 and 20 μM. Bars show the average and error bars show the spread (n=2).

3.6 C1321

3.6.1 Further examination of concentration dependency

To investigate the effect of different concentrations of the Wnt-3A conditioned medium together with different concentrations of compound C1321, HEK293 STF cells were activated with different concentrations of Wnt-3A conditioned medium and compound. Two experiments were set up with final concentration of compound C1321 of 1, 5, 10 and 20 μM , in duplicates. One experiment was set up with final concentration of 5, 10, 20 and 40 μM , also in duplicates. All three experiments had four negative controls with regular cell medium and three positive controls for each medium concentration of Wnt-3A conditioned medium. The positive to negative ratios varied from 2 to almost 600, which is a measure of the strength of the medium (see Figure 30).

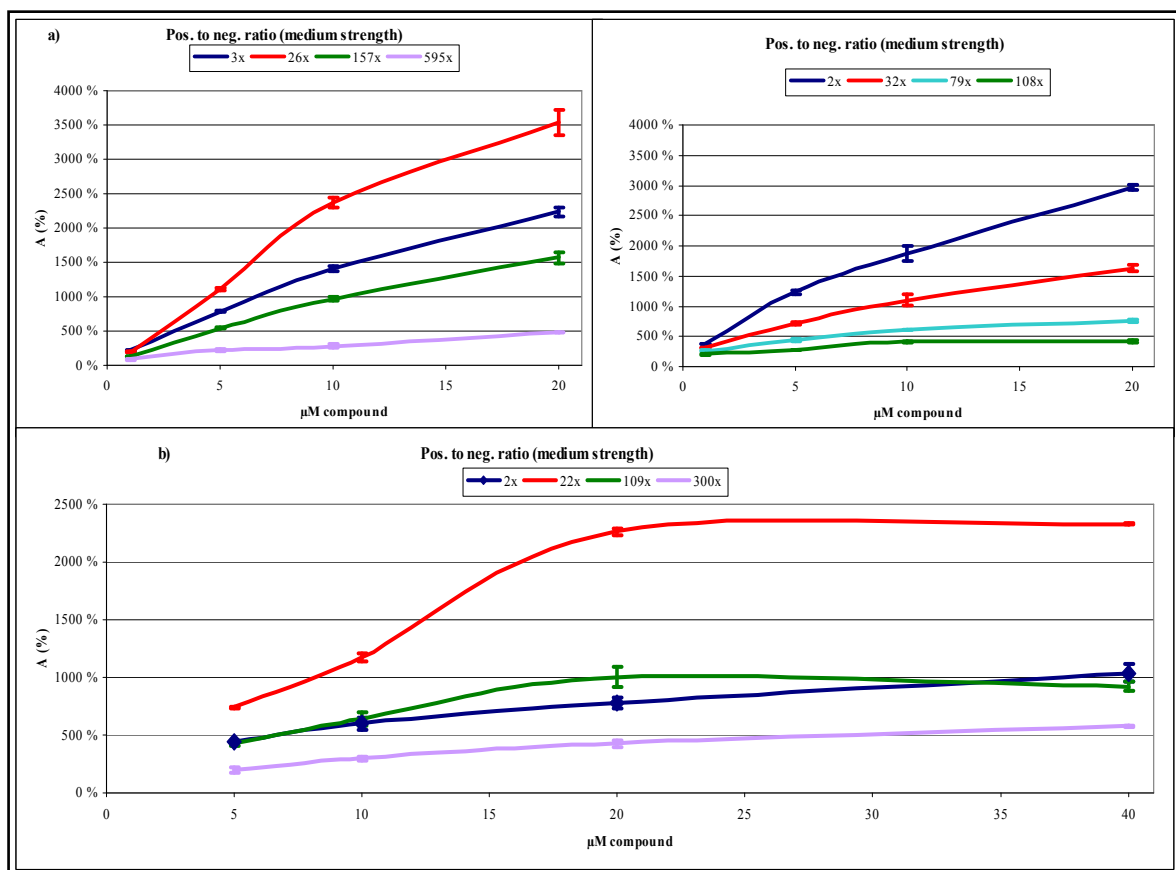


Figure 30 The relative luciferase activity in % (2.10 A) for different concentrations of compound C1321 in HEK293 STF cells activated with Wnt-3A conditioned medium at different concentrations. The trend lines are drawn between average values and error bars show the spread (n=2).

a) Two experiments with added compound to a final concentration of 1, 5, 10 and 20 μM .

b) One experiment with added compound to a final concentration of 5, 10, 20 and 40 μM .

Increased concentration of compound gave an increased luciferase signal for all of the different medium concentrations, however with a slight decrease for 40 μ M of compound with medium with a positive to negative ratio of 109, which was possibly due to toxic or non-specific effects on the cells. A very high positive to negative ratio (strong Wnt-3A conditioned medium) generally gave less luciferase activity compared to more moderate positive to negative ratios. A medium to low positive to negative ratio gave the best luciferase activity, but the luciferase activities varied a lot between the experiments. The varying relative luciferase activity due to varying strength of Wnt-3A conditioned medium could possibly explain some of the variation in relative activity seen for the other compounds as well.

3.6.2 Real-time RT-PCR

Activation of the canonical Wnt signaling pathway will give an increased concentration of β -catenin, which will translocate into the nucleus. After the β -catenin enters the nucleus it functions as a temporary framework to bind TCF/LEF-family transcription factors to the Wnt target genes and trigger a transcription of these genes (see Figure 2).

With pSTF transfected as a plasmid, or stably transfected into the cell line, the luciferase signal will be activated through 7xTCF binding sites (see section 1.7.5).

Axin2 is a well described¹¹⁰ target gene and its transcript is promoted by activated Wnt signaling^{111, 112, 113}. In RT-PCR Axin2 could be used to quantify the activation of the canonical Wnt signaling pathway. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is a gene that is constitutively expressed in the cells, often referred to be a housekeeping gene^{107, 114}, and is often used as an internal control in RT-PCR. A housekeeping gene is generally a constitutive gene which is found in most cells and that controls basic cellular functions, and that give constant expression¹¹⁵.

HEK293 wt cells were plated in a 12 wp coated with poly-L-lysine and the cells were grown overnight. Different concentrations of Wnt-3A conditioned medium were used to activate the canonical Wnt signaling pathway, and C1321 was added to a final concentration of 5, 10 and 20 μ M. Four replicates were performed, and four positive and four negative controls were used.

A real-time RT-PCR with primers for Axin2 and GADPH was performed with standard protocols (see section 2.8), and MxPro QPCR Software from Stratagene was used for calculations.

Figure 31 shows a general increase in activation when C1321 was added, compared to positive and negative controls.

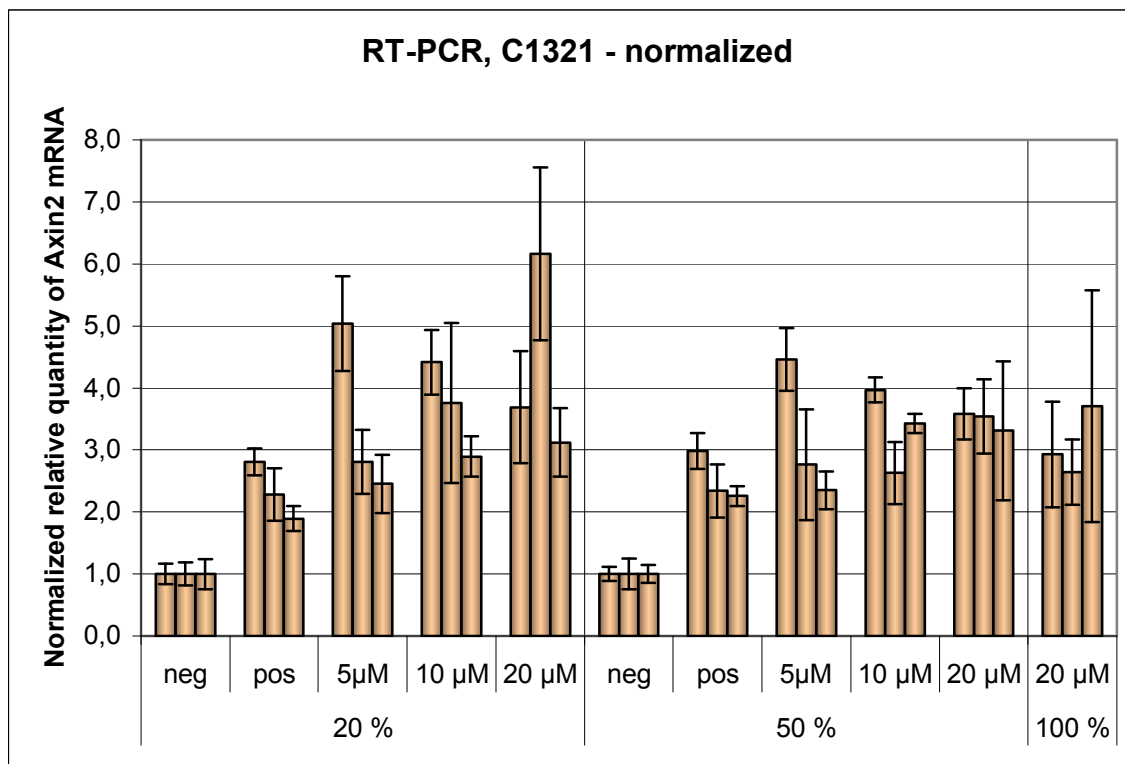


Figure 31 The normalized relative quantity of Axin2 mRNA for compound C1321 in HEK293 wt cells activated with Wnt-3A conditioned medium (20, 50 and 100 %) and added compounds to a final concentration of 5, 10 and 20 μ M. Bars show the average and error bars show the standard deviation (n=4).

Figure 31 shows that compound C1321, in a dose dependent manner, is capable of promoting transcription of the canonical Wnt signaling pathway target gene Axin2. This is consistent with the experiments done on HEK293 STF cells with the Luciferase Assay System.

20 % Wnt-3A conditioned medium obtained a somewhat but not substantially higher quantity of Axin2 mRNA than with 50 % and 100 % Wnt-3A conditioned medium.

4 Final discussion and conclusion

Andrew Voronkov suggested 256 small molecules in an *in silico* based screening to be potential modulators of the Wnt/Frizzled (CRD) interaction. By modulating this interaction, we wanted to increase or decrease the canonical Wnt signaling activity. These 256 compounds were investigated, in a small based screening experiment, against a luciferase based reporter system in the Wnt-3A responsive cell line HEK293 STF. Twelve potential inhibitors and 33 activators (synergists) were found based on defined threshold levels. The inhibiting activities of three compounds (C1270, C1290 and C1336) were confirmed by a second experiment setup. However, these three compounds also affected endogenous luciferase activity and only a minor signal reduction was obtained when a *Renilla* internal reporter was used in parallel. As the potency of the inhibitors was ineffectual, when compared to previously published inhibitors^{73, 75}, no further efforts in development of the chemotypes were pursued. Seven of the 33 activators (C1104, C1109, C1110, C1129, C1132, C1321 and C1344) were likewise chosen for further investigation based on the activating capacity in a second experiment. No substantial decrease of the endogenous luciferase activity was detected after exposure by these seven compounds on HEK293 STF cells. The seven compounds did not activate the pathway without Wnt-3A present in the cell culture medium. This indicates that the compounds affect the activity of canonical Wnt pathway only when the Wnt/Frizzled (CRD) complex is formed. An enhanced complex interaction, promoted by the compounds, may therefore be suggested. The specific effect on the Wnt/Frizzled interaction was supported as none of the seven lead compounds could activate or synergize the activity of Shh- or NF- κ B luciferase reporters, which are both pathways that also are activated by ligand to receptor binding. To further develop the potency in synergizing the canonical Wnt signaling pathway, and the affinity against the Wnt/Frizzled complex, 226 analogues based on the seven lead compounds were suggested by Andrew Voronkov. No enhanced activating properties could be detected among these analogues when compared to their originating chemotypes. Compound C1321 was identified to be the compound with strongest capacity in synergizing the canonical Wnt signaling pathway in HEK293 STF cells. The specific activity of compound C1321 was further confirmed by real-time RT-PCR measurement of the Wnt target gene Axin2.

Collaboration with Dietmar Gradl (University of Karlsruhe) showed that compound C1321 had a similar synergizing effect on development of a second body axis after co-injection in a *Xenopus* axis duplication assay (data not shown). A second body axis is formed in *Xenopus* when XWnt8 is injected into the ventral blastomeres of a four-cell stage embryo¹¹⁶.

Taken together, the compound C1321 activity on HEK293 STF reporter activity, Axin2 real-time RT-PCR and *Xenopus* axis duplication indicate the promising potential in modulating the canonical Wnt signaling pathway at the receptor level. Compound C1321 has a stronger synergizing effect in the STF assay when compared to the previously published compound QS11⁷¹. The stability and the target affinity may be further developed by suggesting new analogues to enhance the C1321 synergy efficacy. Compound C1321 should also be examined against other cell lines and especially against stem cells (adult and embryonic) and cells from the neuronal lineage. If efficacy is shown against stem cells or neuronal cells, compound C1321 may have potential in controlling stem cell differentiation¹¹⁷ and protect against Alzheimers disease¹⁷.

Attachments

Attachment 1: Recipes

PBS:

137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, PH 7,4

- Dissolve in 800 mL of distilled H₂O
- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na₂HPO₄
- 0.24 g KH₂PO₄
- Adjust the pH to 7.4 with HCl
- Add H₂O to 1 L
- Sterilized by autoclaving (20 min, 121 °C, liquid cycle)
- Stored at room temperature

TE buffer:

- 10 mM Tris
- Adjust to pH 7.5 with HCl
- 1mM EDTA

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